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The Nitrogen Metabolism of Gram-positive Bacteria. ERNEST FREDERICK GALE (The Herter Lectures for 1948)

I. The Assimilation of Amino-Acids 119

II. Intracellular Utilization of Glutamic Acid and its Inhibition by Certain Antibacterial Agents 134

III. The Nature of Penicillin Sensitivity in Staphylococcus Aureus 154

Sulfhydryl Compounds and the Sickling Phenomenon. A Preliminary Report. LEWIS THOMAS AND CHANDLER A. STETSON, JR. 176

Book Reviews 181

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THE NITROGEN METABOLISM OF GRAM-POSITIVE BACTERIA*

ERNEST FREDERICK GALE

Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, Cambridge, England

I. THE ASSIMILATION OF AMINO-ACIDS
II. INTRACELLULAR UTILIZATION OF GLUTAMIC ACID AND ITS INHIBITION BY CERTAIN ANTIBACTERIAL AGENTS
III. THE NATURE OF PENICILLIN SENSITIVITY IN STAPHYLOCOCCUS AUREUS

LECTURE 1

THE ASSIMILATION OF AMINO-ACIDS

The growth of any cell involves an increase in its parts. One of the most important constituents of the bacterial cell is protein and the growth of a bacterial cell involves the synthesis of a wide variety of structural and functional proteins within the cell. Analysis of the amino-acid composition of bacterial proteins (1, 2) shows that these proteins resemble those of all other cells in being built up from some twenty-odd different amino-acids. Consequently the synthesis of protein by the growing cell requires the provision of all the necessary amino-acids in the correct proportions. Such provision can be made in one of two ways: by synthesis, or by assimilation of the ready-formed substances from the environment. The synthetic abilities of bacteria can be tested by investigation of their nutritional requirements, and such investigations reveal wide differences between various species and strains. The autotrophic organisms can synthesise all their amino-acids from very simple inorganic substrates such as CO_2 and ammonia; some heterotrophic bacteria can synthesise all their requirements from ammonia as N-source and a single C-source such as glucose but other bacteria of the heterotrophic group cannot grow unless they are supplied with certain amino-acids in the ready-formed state. The synthetic disability may be simple, such as that of *Eberthella typhosa*

* Three Herter Lectures delivered at the Johns Hopkins Hospital, March 22–24, 1948.

which requires tryptophan, or complex such as that of *L. casei* or *Strep. haemolyticus* strains of which may require as many as 20 amino-acids. In these cases synthesis of certain amino-acids is impossible and growth—*i.e.* protein synthesis—can occur only if the organisms can assimilate the essential amino-acids from their external environment.

A number of workers have turned their attention to the problem of how these nutritionally-exacting organisms have arisen. Their present nutritional requirements restrict their habitat to environments rich in amino-acids and, in many cases, growth factors; environments which are to be found naturally only in biological fluids. Knight (3) and Lwoff (4) are among those who have suggested that the exacting organisms have developed from non-exacting ones; that if a non-exacting organism assumes a parasitic existence or becomes established in a biological fluid then growth may occur more rapidly by the utilisation of essential substances already present in the environment than by synthesis of these substances, and that the ability to synthesise such substances will then be gradually lost through disuse. Weight is lent to this hypothesis by the demonstration that non-exacting organisms can be derived from exacting ones if the latter are treated to a process of attrition. Thus if *Eberthella typhosa* is subcultivated serially into media containing progressively less and less tryptophan it will eventually be able to dispense with added tryptophan and synthesise its complete requirement (5), while strains of *Staph. aureus* initially needing ten or more amino-acids can, by a similar process, be trained to synthesise all their amino-acid requirements from ammonia (6).

If the ability to synthesise an amino-acid is lost, then the organism must be able to assimilate that amino-acid in order to grow. It is with this process of assimilation that I wish to deal mainly today. In essence the process involves the removal of an amino-acid from the external environment and its passage into the cell into the region where its further metabolism may be accomplished.

The work started, as so much scientific work does, from an investigation into a little-related problem. In the course of our studies at Cambridge into the amino-acid metabolism of bacteria, we found that some bacteria grown under certain conditions are able to produce enzymes which quantitatively and specifically decarboxylate certain amino-acids to their corresponding amines (7). These enzymes, which

can be prepared in a specific state and whose action can be studied easily by the manometric method, provide an accurate and rapid micro-method for the estimation of their substrates (8, 9). In each case the enzyme will attack the specific amino-acid substrate only provided that three polar groups are free: the —COOH group, the alpha-NH_2 group, and a third polar group remote from the —COOH group attacked; in consequence the enzymes estimate the free amino-acid substrate only. We were using these enzymes to investigate the amino-acid composition of a variety of bacteria by analysis of acid-hydrolysates of washed suspensions of the organisms concerned. It occurred to us that a certain amount of free amino-acid might in some cases be carried down from the growth medium either on the outside or within the cell suspensions and that this would invalidate values obtained after acid hydrolysis. To check whether this was the case, we divided our cell-suspensions into two parts, one of which was subjected to acid hydrolysis as usual and the other was disintegrated by mechanical agitation with glass particles. The two preparations were then analysed by the decarboxylase method. Results fell into two groups; in the first group, which comprised organisms such as *Escherichia coli* and *Aerobacter aerogenes*, the amount of free amino-acid found in the disintegrate was negligible when compared with that obtained after hydrolysis; in the other group, which comprised organisms such as *Staph. aureus* and *Strep. faecalis*, an amount of free amino-acid was found in the disintegrated cell which amounted in some cases to as much as 50% of the total amino-acid liberated after hydrolysis.

The position is made clear by fig. 1. In this case we take a thick suspension of well-washed cells of *Strep. faecalis* grown in a casein-digest medium and use it as substrate for glutamic acid decarboxylase; it can be seen that the addition of the enzyme is followed by a small evolution of gas (carbon dioxide) which corresponds to free glutamic acid carried down with and on the outside of the cells during harvesting and washing. If, however, an equivalent sample of cells is mechanically disrupted, then the glutamic acid assay is greatly increased. This extra glutamic acid must be liberated by the disruption of the cell-wall. Similar results are obtained if the cell-wall is broken by heat denaturation or by treatment with detergent substances (10, 11).

At this point it may be useful to define the terms in which concentra-

tions of amino-acids, etc. will be stated and discussed in these lectures. All estimations of amino-acids have been made by the manometric method in which the evolution of CO_2 equivalent to the amino-acid present is measured; consequently it is convenient to discuss the results in terms of the gas evolution in each case i.e. in μl. This means that 1 m.mol. of amino-acid is equivalent to 22.4×10^3 μl. or, alternatively, concentrations can be converted to molecular quantities such as μmols. by division of the values by 22.4.

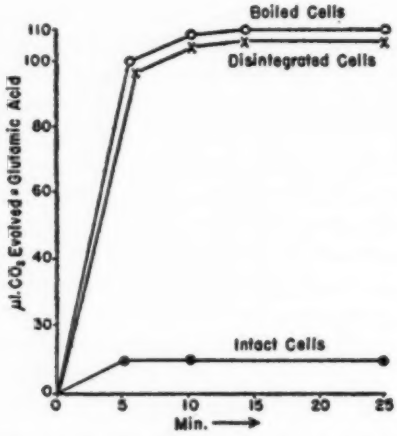

FIG. 1. LECTURE I. Action of glutamic acid decarboxylase on intact and disrupted cells (*Strep. faecalis*). Each estimation carried out on 60 mg. dry weight of cells.

The experiment just described demonstrates that cells of *Strep. faecalis* contain free glutamic acid in their internal environment; 100 mg. dry weight of cells contain approx. 170 μl free glutamic acid. Similar investigations with other enzymes of the decarboxylase series show that the cells also contain lysine, histidine and ornithine in the free state within the internal environment but, in *Strep. faecalis*, no free arginine or tyrosine. The absence of these last two amino-acids is probably due to the fact that the cells possess enzymes which bring about their rapid decomposition—arginine dihydrolase and tyrosine decarboxylase. Other amino-acids almost certainly exist in the free

state within the cell but absence of suitable techniques for their estimation has made it impossible to investigate this possibility up to the present. A question that immediately arises is—can free amino-acids be demonstrated within the internal environment of all cells? You will remember that, in the early investigations on amino-acid composition, some cells contained free amino-acids and others did not. Consequently we started an investigation of a variety of organisms; they were all grown in casein-digest (as a growth medium rich in free amino-acids) with glucose and such specific growth substances as might be required by exacting organisms; they were then well-washed and their

TABLE 1 (LECTURE I)

Internal Concentration of Glutamic Acid

Values expressed in μl. glutamic acid (internal)/100 mg. cells

Staph. aureus 563	820	*Esch. coli* 86	0
Staph. aureus 6773	635	*Esch. coli* EST	0
Staph. aureus D	456	*Esch. coli* 7020	0
Strep. faecalis ST	230	*Aerobact. aerogenes* I	0
Sarcina lutea	225	*Aerobact. aerogenes* II	0
M. lysodeikticus	203	*N. catarrhalis*	0
L. casei YCT	174	*B. brevis*	0
Strep. faecalis SF	107	*Proteus vulgaris*	0
Strep. haemolyticus R	99	*Ps. pyocyanea*	0
Cl. sporogenes Bellette	81	*Bact. paracoli* 6578	0
L. delbreuckii B	80	"Staph. PT"	0
L. helveticus B	74		
B. subtilis	20		
B. mesentericus	14		

lysine and glutamic acid content investigated before and after rupture of the cell-wall. Table I shows results obtained for the free glutamic acid content (12). The organisms fall into two well-defined groups; those which possess free amino-acids in the internal environment, and those which do not. A further correlation is immediately obvious; all those cells in the first group are Gram-positive while those in the second group are Gram-negative and no exception to this rule has yet been discovered. The ability of the Gram-positive cells to accumulate free glutamic acid varies widely; within our experience the species which can, in general, effect the highest internal concentration of glutamic acid is *Staph. aureus*. At the other end of the scale we get organisms

such as *B. subtilis* and some of the *Clostridia* which contain small but still definite amounts of free glutamic acid within the cells. The yeasts, also Gram-positive, are interesting in that they accomplish a high concentration in the internal environment of all six of the amino-acids that we can study by this method. But however it is grown or tested, and we have tried many things, it has not yet been possible to demonstrate any free amino-acid inside the Gram-negative cell.

Most of the work I shall be describing to you has been carried out either with *Strep. faecalis* or with *Staph. aureus*, both of which can effect a high concentration of both glutamic acid and lysine but, as we shall see later, apparently do so by different mechanisms. The existence of the free amino-acid within the cell in these cases enables us to investigate the metabolism of that free amino-acid by a study of the conditions which affect the level of the free amino-acid within the cell.

In the first place, the level within the cell depends to a certain extent on the level in the external environment. The estimations described so far were carried out on cells which had been grown in an external medium rich in amino-acids. The quantity of amino-acid in the medium can be cut down considerably without preventing growth and a reasonable amount of organism can be obtained in a medium containing 0.1% Marmite as sole source of amino-acids. Such a medium we call our "deficient" medium although it is not deficient in that any particular growth essential is missing—they are merely there in minimal quantities rather than in gross excess as in the casein-digest medium. *Strep. faecalis* cells grown in this deficient medium have much less free glutamic acid or lysine within the cells than when they are grown in the rich medium. This then enables us to establish a useful technique: by growing our organisms in a deficient medium we can produce cells which possess little free amino-acid in the internal environment; by taking such cells and placing them in an external environment rich in free amino-acid we can investigate the conditions whereby such amino-acids pass across the cell-wall and accumulate within the cell. Alternatively we can grow cells in an amino-acid-rich medium, place them in an amino-acid-free environment and study the conditions under which the internal amino-acids will pass out of the cell.

Let us take the second case first, since it might affect the conditions of our estimations. If we take cells containing a high internal concen-

tration of either lysine or glutamic acid and stand them in either distilled water or a suitable salt solution at 0° or 37° then neither lysine nor glutamic acid can diffuse out of the cell unless autolysis takes place. This holds true over the pH range 5.5–8.5 although some leakage occurs outside that range—possibly due to damage to the cell-wall under these conditions. Our amino-acid estimations by the manometric method take about 15. min. so that the technique described for the estimation of internal and external amino-acids is not invalidated in any way by leakage across the cell-wall during estimation.

Now let us turn to the opposite aspect—the migration of amino-acids from external to internal environment. I intend to deal with two amino-acids only in detail—lysine and glutamic acid. Substances can pass across a cell-wall by two processes; either by diffusion or as a result of an active process by the cell. The evidence suggests that lysine passes across the cell-wall by diffusion while glutamic acid enters the cells as part of an active process (11).

If cells deficient in lysine are placed in a solution of lysine in a salt medium, then lysine begins to enter the cell immediately the latter comes into contact with its new environment and enters the cell very rapidly until the new level inside is attained. In the case shown the experiment was carried out at 10° and the new level within the cell is attained within 10 min. of contact. The migration occurs equally well at 0° and is unaffected by any of the common inhibitors of metabolism such as HCN, iodoacetate, etc.

If deficient cells are placed in a solution of glutamic acid or glutamine, no entry into the cell takes place even at 37° over a period of 3 hr. If glucose is added to the external medium then fermentation takes place and glutamic acid (or glutamine) enters the cell until a new level in equilibrium with the external environment is reached. *Strep. faecalis* cells have restricted catabolic activities but can also attack arginine by the enzyme arginine dihydrolase (13) and it appears that arginine can also act as a source of energy for the migration of glutamic acid although its efficiency as energy source is much less than that of glucose. The migration in the presence of glucose is inhibited by iodoacetate or sodium fluoride in concentrations inhibitory to glycolysis. Glucose can, under certain conditions, be substituted by adenosine-tri-phosphate as energy source.

The difference between the two processes can be further shown if we study the rates at which the two amino-acids enter deficient cells. The rate of entry of lysine is roughly proportional to the external concentration over the range studied, whereas the rate of entry of glutamic acid reaches a maximum when the external concentration approaches 50 μl./ml. The curve obtained for glutamic acid is similar to that obtained for the variation of the rate of an enzyme reaction with substrate concentration while that obtained for lysine is consistent with a diffusion process in which the driving force consists of the difference in concentration between internal and external environments.

The effect of temperature also differentiates the two processes. The rate of entry of lysine increases linearly with temperature and the temperature coefficient over the range 20–30° is 1.40 which is within experimental error of the value obtained for the free diffusion of lysine. The rate of entry of glutamic acid varies in a completely different manner with temperature and the temperature coefficient of approx. 2 over the range 20–30° is again typical of an enzymatic reaction.

It would be of considerable interest to know which amino-acids other than lysine and glutamic acid enter the cell by diffusion or by an active process but we are at present handicapped in such a search by the absence of really suitable methods of investigation for most other amino-acids. We can differentiate between those amino-acids which can pass into washed deficient cells at 37° in the absence of a source of energy and those which can only do so in the presence of a source of energy such as glucose. Histidine passes into the cell to a limited extent in the absence of glucose and to a markedly greater extent in the presence of glucose. Aspartic acid, as judged by the rather non-specific chloramine-T estimation, only enters the cell as a result of an active process. Arginine cannot be shown to enter the *Strep. faecalis* cell at all by this method—probably because arginine dihydrolase removes any accumulated excess. Of the amino-acids that we can estimate, only lysine appears to enter the cell by simple diffusion. The same general findings hold good for *Staph. aureus*; lysine enters by diffusion, glutamic acid as the result of an active process. In yeasts we find that all the amino-acids tested, including lysine, can enter the cell only while glucose is being metabolised and all the amino-acids, including arginine and tyrosine, accumulate at high concentration within the internal environment (14).

Returning again to *Strep. faecalis* we find that both lysine and glutamic acid enter the cell, under suitable conditions, until the internal concentration is markedly higher than that holding in the external environment. By direct measurement of the volume of the cell and calculation of the free space within the cell, we can get some idea of the ratio between internal and external concentrations. In the case of lysine the internal concentration in *Strep. faecalis* varies from 2–3 to 15–20 times the external concentration over the range studied (25–400 μl./ml.); the lower the external value, the higher the relative concentration within the cell. In the case of glutamic acid, the ratios reach 50–60 in low external concentrations while the actual concentration within the cell may reach 0.06*M* glutamic acid. These values may be considerably exceeded in the case of some strains of *Staph. aureus* which have greater capacity for the internal concentration of glutamic acid and where an internal concentration of 0.18*M* free glutamic acid has been measured and a ratio of internal to external concentration of 400 obtained.

The entry of both lysine and glutamic acid into the cell is made against the concentration gradient. Biophysicists tell us that there are two conditions under which such a migration can occur:

1. That the cell-wall is permeable to the substance concerned and that this is held by electrical or chemical combination on the side of greater concentration.

2. That the cell-membrane is impermeable to the substance concerned and that migration across the membrane takes place in a different physical or chemical state from that which accumulates on one side.

The evidence we have considered so far indicates that lysine diffuses freely into deficient cells but does not pass out of these cells if they are placed in a lysine-free medium. Under physiological conditions lysine is positively charged while the bacterial cell is negatively charged; consequently there is an electrical attraction between the cation and the cell. It seems probable that lysine enters the cell by diffusion and is held therein by electrical combination. We have attempted to get some light on this point by investigations of the electrophoretic mobility of the cells in the presence and absence of lysine (15) and it has been possible to show that there is a definite decrease in the negative surface charge of deficient cells when brought into contact with lysine.

However there is other evidence that the situation is not as simple

as this. You will remember that lysine enters the deficient *Strep. faecalis* cell at 37° in the absence of glucose; if glucose is added to the system during assimilation then the entry of lysine is reduced and the level attained within the cell may be reduced to 10% of that attained in the absence of glucose. Likewise if cells saturated with lysine are incubated in water, no outward diffusion of lysine takes place; if however glucose is added to the external medium a rapid outward diffusion takes place until the internal level is adjusted to a lower level. If the lysine is held within the cell by a negative charge, it would appear that the presence of glucose has had the effect of discharging the cell; electrokinetic measurements however show that the negative charge on the surface of the organism is not affected by the presence of glucose. We do not know, of course, what is happening within the cell but it would seem that, if the lysine is in fact held by an internal negative charge, then that charge must arise from some specific centre—such as an ion which migrates out of the call during glycolysis—rather than the simple net charge on the cell as a whole.

If we study the effect of the pH of the external medium on the rate of entry of lysine into the cell, we get a curve showing that the rate increases with rising pH to the limit of alkali tolerance at pH 9.5. Since the isoelectric point for lysine is 9.47 it appears that the amino-acid crosses the cell-wall most readily in the isoelectric state. The final concentration attained within the cell is not markedly affected by the external pH, the level attained at an external pH 8.5 being 14% higher than that attained at an external pH 5.0. It seems probable that lysine passes across the cell-wall in an isoelectric state but accumulates within the cell in the cationic state. It is obvious that we need much more knowledge concerning the ionic balance within the cell and the ionic exchanges that occur during metabolism before we can obtain any true understanding of lysine assimilation.

If we turn to the assimilation and internal accumulation of glutamic acid, we have to admit that the position is even less satisfactory. This amino-acid enters the cell not by diffusion but as a result of some energy-requiring process. The amino-acid exists in an anionic state in physiological conditions and is therefore entering the cell against the electrostatic gradient and against the concentration gradient. Electrophoretic measurements show that the surface charge on deficient *Strep. faecalis* cells is unaffected by the presence of glutamic acid either

alone or in the presence of glucose although assimilation will take place in the latter case. These negative results lend weight to the electro-diffusion hypothesis for lysine assimilation where the addition of lysine is followed by a definite reduction in the charge on the organism. With *Strep. faecalis* cells, the internal and external glutamate concentrations are in true equilibrium as long as a source of energy such as glucose is present *i.e.* if deficient cells are incubated in high concentrations of glutamic acid, then the amino-acid enters the cell until equilibrium is established; if saturated cells are incubated in glutamic-acid-free media, then glutamic acid leaves the cell until equilibrium is established and, for a given external concentration, the same internal concentration is reached whether the amino-acid is passing into or out of the cell. No diffusion in either direction takes place in the absence of exergonic metabolism. It would seem that we may have here a case where the cell-wall is impermeable to the substance concentrated within the cell—and, it may be, that the substance crossing the cell-wall is not glutamic acid itself.

The first possible migrant substance we tried was glutamine. There is a certain amount of evidence that this may be involved. If the rates at which glutamine and glutamic acid pass into the deficient cell are studied, then glutamine is often found to pass more rapidly while glutamic acid may show a short lag phase after introduction of the cells before it begins to enter the cells. But glutamine again does not give rise to an internal concentration in the absence of glucose; in the presence of glucose, glutamine and glutamic acid give rise to the same internal concentration. Estimations of the nature of the internal substance under such conditions indicate that it is a mixture of glutamic acid and glutamine containing 20–40% glutamine. It has been shown recently in this country by Waelsh *et al.* (16) that methionine sulphoxide competitively blocks the utilisation of glutamic acid by certain *Lactobacilli* and is non-competitively neutralised by glutamine. This suggests that methionine sulphoxide inhibits the formation of glutamine from glutamic acid. Elliott (17) has now shown that the first stage in the synthesis of glutamine involves a phosphorylation of glutamic acid, in the presence of adenosine-tri-phosphate, and we (18) have shown that the enzyme concerned is competitively inhibited by methionine-sulphoxide.

The assimilation of glutamic acid by *Strep. faecalis* requires glucose

but will also take place if large amounts of adenosine-tri-phosphate are added (approx. 30–50 molecules of ATP are required to promote the assimilation of 1 molecule of glutamic acid). Further, the assimilation in the presence of either glucose or ATP is competitively inhibited by methionine sulphoxide while that of glutamine is unaffected. This clearly suggests some role of glutamine or a like substance in glutamic acid assimilation. However glutamine itself still requires either glucose or ATP to migrate across the cell-wall and would appear to be ruled out as the migrant substance. But a phosphorylated derivation of glutamic acid may be an intermediate step in the synthesis of substances other than glutamine - glutathione and vitamin B_c conjugate appear probable for example. Glutathione has been tested as the migrant substance but does not give rise to either glutamic acid or glutamine inside the cell and our knowledge of the impermeability of cell-membrane towards acyl-phosphates would suggest that "glutamyl-phosphate" itself could not penetrate into the cell. There is, however, definite evidence pointing to the idea that a metabolite of glutamic acid is the form in which this amino-acid crosses the cell-wall.

However, when we try to extend these results to other organisms we find that we do not always get the same picture. The process of glutamic acid assimilation in *Staph. aureus* possesses several features which suggest that it is different from that in *Strep. faecalis*. Glutamic acid does not pass into deficient *Staph. aureus* cells in the absence of a source of energy such as glucose. In the case of *Strep. faecalis* no leakage of internal glutamic acid takes place in the absence of glucose while, in the presence of glucose, there is a migration across the cell-wall in either direction until equilibrium is established. In the case of *Staph. aureus* however there is a slow leakage of glutamic acid out of the cell in the absence of glucose and the addition of glucose checks rather than enhances this leakage—the picture being that the glycolysis is holding the amino-acid inside the cell in opposition to the outward diffusion process rather than establishing a new equilibrium across the cell-wall. In the case of *Strep. faecalis*, methionine sulphoxide competitively inhibits the assimilation process; in *Staph. aureus* it has no effect on assimilation even when present in a concentration 200 times that of the glutamic acid in the external medium.

A possibility which we have not considered yet is that of ionic

exchange. A secretion process may sometimes consist of the active transfer of one ion across a membrane to substitute for another ion of like charge which passes across the membrane in the opposite direction. It may be that glutamic acid passes into the cell replacing some other negative ion which passes out of the cell. Hotchkiss (19) has shown that *Staph. aureus* cells accumulate phosphate within the cell in high concentration and that the amount of phosphate within the cell is reduced if amino-acid assimilation takes place. He points out that during glycolysis an uptake of both nitrogenous and phosphate compounds takes place but that as more amino-acids are added to the external medium—as the medium becomes more complete in the nutritional sense—then more nitrogenous material is taken up and less phosphate. This might mean that glutamic acid, and possibly other amino-acids, enter the cell in replacement of phosphate ions. However, phosphate is involved in so many reactions within the cell and, as McIlwain (20) has shown, glycolysis itself is increased after assimilation of glutamine so it is difficult to interpret these results with any certainty at present.

The last question with which I wish to deal today is the nature of the boundary in the cell which holds the internal concentration of amino-acid and maintains the difference in concentration between internal and external environments. The work of Hotchkiss (21) on the action of the antibiotic Tyrocidin and of other detergent substances enabled us to obtain some information on this point. Hotchkiss observed that if cells of *Strep. faecalis* or *Staph. aureus* were treated with such detergents, then nitrogenous and phosphorus-compounds were liberated into the external medium. It seemed highly probable that the nitrogenous material liberated would include these amino-acids which were held within the cell. Fortunately it proves very easy to demonstrate this fact since the amino-acid decarboxylase preparations are not inhibited by high concentrations of tyrocidin and other detergents.

This makes it possible for us to use the manometric method to study the action of tyrocidin: a thick washed suspension of cells is placed in the main cup of the manometer and glutamic decarboxylase added from one side-bulb at the beginning of the experiment. When the external glutamic acid has been assayed and removed, tyrocidin

is added from a second side-bulb and the release of glutamic acid from the cell can be seen to start immediately. The total internal free amino-acid can be judged from the assay with boiled cells and it can be seen that complete lysis occurs within 5–10 mins. of the addition of the tyrocidin (11).

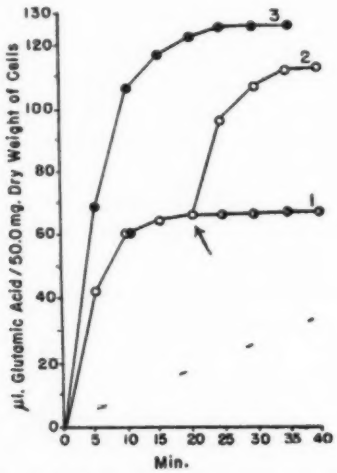

FIG. 2. LECTURE I. Relation between quantity of tyrocidin added and amount of glutamic acid released from internal environment. Dry weight of *Strep. faecalis* cells assayed = 50.0 mg. Temperature = 30°. Manometer vessels made up with 1.0 ml. M/5-phosphate pH 6.0; 1.0 ml. washed suspension of *Strep. faecalis* cells, and 0.5 ml. glutamic decarboxylase (main compartment) with tyrocidin in side-bulbs. Manometers allowed to equilibrate and liberation of external glutamic CO_2 to cease before time = 0. Curve 1: 0.5 mg. tyrocidin tipped at time = 0. Curve 2: 0.5 mg. tyrocidin tipped at time = 0; further 0.5 mg. tyrocidin tipped at time = 20 min. (arrow). Curve 3: 1.0 mg. tyrocidin tipped at time = 0.

A very interesting thing is the effect of detergent concentration; if an amount of tyrocidin is added equal to half that required to liberate all the amino-acid, then we find that only half the total amino-acid is liberated—not, as one might expect, that all the amino-acid is liberated at half the speed. If we add a second quantity of tyrocidin after the effect of the first has finished, we find that the rest of the amino-acid is liberated. These results can be correlated with viable

counts: if an amount of tyrocidin is added sufficient to release all the amino-acid, then all the cells are killed; if half this quantity is added, then half the cells are killed. From quantitative considerations it can be calculated that about 10^8 molecules of tyrocidin are required to kill one *Strep. faecalis* cell.

The action of these detergents is apparently to modify the cell-wall in such a way that the concentration boundary is broken. The electron microscope has long revealed that most bacteria have a definite cell-wall although this is not easy to demonstrate in some of the Gram-positive cocci. However, electron-micrograph studies of *Strep. faecalis*, *Staph. aureus* and yeast cells before and after treatment with tyrocidin show clearly that the action of the detergent is to strip off the outer cell-wall (22). The cells are not completely lysed as they retain their shape and under the dark-ground appear little altered apart from a slight change in the clarity of the outline. This suggests either that there is some containing-membrane within the cell-wall which is not broken by the detergent action, or, alternatively, that the cytoplasm of the cell is in a gel form.

Phenol releases amino-acids from the cells in the same manner as tyrocidin although the action in this case is much slower and electron-micrographs again demonstrate a stripping of the cell-wall after phenol-action. We have endeavoured to determine the structure of the material stripped from the cell by phenol—using phenol since it is easily and quantitatively removed from the preparation by drying *in vacuo*. The material obtained by phenol treatement of large amounts of *Strep. faecalis* contains about 50% nitrogenous material consisting largely of free amino-acids released from inside the cell, some phosphorous-compounds also largely released from the cells and approximately 40% ether-soluble, saponifiable material which probably comes from the cell-wall itself.

To summarise our main conclusions from the work studied so far; we find that Gram-positive bacterial cells assimilate and accumulate certain amino-acids in the free state within the internal environment; that some amino-acids, notably lysine, pass into the cell by diffusion but others, notably glutamic acid, can only enter the cell as a result of an active process; that the equilibrium concentration attained within the cell is markedly greater than that holding in the external environ-

ment, and that this concentration gradient is maintained at the cell-surface by a cell-wall probably consisting largely of lipoid substances which can be dissolved away from the cell by certain detergent substances.

In the next lecture, we shall proceed to discuss what happens to the glutamic acid after assimilation and concentration within the Gram-positive cell.

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LECTURE 2

THE INTRACELLULAR UTILIZATION OF GLUTAMIC ACID AND ITS INHIBITION BY CERTAIN ANTIBACTERIAL AGENTS

Glutamic acid appears to occupy a key position in microbial metabolism. It forms the main substrate for the transaminase systems

demonstrated in bacteria (1, 2) and, as such, is involved in the synthesis of aspartic acid and alanine. Bonner (3) has demonstrated by means of the biochemical mutant technique that it is the biosynthetic precursor of proline, ornithine, citrulline and arginine in *Penicillium*. Synthesis of the *L. casei* factor has shown this to be pteroylglutamic acid (4) which is one of a group of substances, known as the folic acid complex, which differ from each other in the number of glutamyl-residues they contain; thus the *casei* "fermentation factor" is pteroyltriglutamic acid and vitamin B_c conjugate contains pteroylheptaglutamic acid. Ratner, Blanchard & Green (5) have isolated a peptide from yeast which contains p-aminobenzoic acid and 10 or 11 glutamyl-residues in addition to an unidentified amino-acid. Glutamic acid forms part of the structure of Glutathione, discovered by Hopkins (6) and which has been demonstrated to act as a growth essential for *N. gonorrhoeae* (7). It is probably one of the constituents of Strepogenin (8) and forms 8–9% of the dry weight of bacterial protein (9).

In the first lecture I showed that glutamic acid is assimilated by Gram-positive bacteria and is concentrated in the internal environment. There is no doubt but that the glutamic acid within the cell must enter into metabolism of the cell and since our method of estimation assays only the free unsubstituted amino-acid, it follows that the level of glutamic acid measured within the cell must represent a balance between the rate at which it is assimilated from the external environment and the rate at which it is metabolised in the internal environment. If further follows that if the cell is treated in such a way as to inhibit the internal metabolism, then the level of glutamic acid will rise within the cell until it reaches the value which represents true equilibrium with the external environment.

When a survey of the action on assimilation of a variety of antibacterial substances was undertaken, it was found that the presence of crystal violet, or other basic dyes of the triphenylmethane series, resulted in a marked rise in the internal level of glutamic acid during assimilation by washed *Strep. faecalis* cells under otherwise standardised conditions.

The development of the dyes of the triphenylmethane series arose from a suggestion made by Paul Ehrlich, the father of chemotherapy

and the first Herter lecturer. He was struck by the specific staining affinities for particular tissues displayed by certain dye-stuffs and suggested that it should be possible to obtain dyes which would specifically stain, and thereby inactivate, parasitic protoplasm without affecting the host protoplasm. The suggestion was followed up by several laboratories and led indirectly to the development of the sulphonamides, through Prontosil, and to Bayer 205 (suramine). It did not reach any resounding success in the field of dye-stuffs proper although dyes of the triphenylmethane series have had limited clinical application.

The fundamental structure of the series is the tri-phenylmethane nucleus with either di- or tri-amino-substitution, acidic derivatives having no antibacterial activity. The carbinol base has no biological activity but if it is heated with hydrochloric acid, a quinenoid derivative is produced which is coloured and has antibacterial action. If the dye is reduced to the leuco-base, which is colourless, the activity is lost. Anti-bacterial activity thus appears to be associated with the quinenoid form and may be associated, as Kumler (10) has suggested, with the resonance that occurs in this form. The simplest dye of the series is p-rosaniline or triaminotriphenylmethane; substitution of alkyl groups in the amino-groups leads to a series of dyes of varying biological activity. The first studies of the series were by Kligler (11) who showed that the antibacterial activity increases as the degree of alkyl substitution increases. Thus crystal violet with six methyl-groups is about 100 times as effective as p-rosaniline with no methyl-groups, but the further substitution of a seventh methyl-group to form a quaternary-N compound, methyl green, results in loss of all biological activity. The varying activities are shown in Table 1 for the case of *Strep. faecalis* as test organism; the increasing activity with increasing alkyl substituion is shown for both tri- and di-amino-triphenylmethane series; the most active dye of those tested is Brilliant Green which contains four ethyl substituents.

Fig. 1 shows the effect of crystal violet on the assimilation of glutamic acid by *Strep. faecalis* cells. The cells were grown and tested as I described yesterday and the diagram shows the rate of appearance of glutamic acid within deficient cells when brought into contact with a high concentration of glutamic acid in the presence of glucose with

or without Crystal Violet. In the presence of the dye we find that the rate of uptake is slightly slower than in the untreated cells but that the final level is markedly higher in the dyed cells than in the untreated

TABLE 1 (LECTURE II)

Inhibition of growth of Strep. faecalis *by dyes of the triphenylmethane series*

NAME	SUBSTITUENT GROUPS	CONCENTRATION OF DYE							
		1/10,000	1/50,000	1/100,000	1/200,000	1/300,000	1/500,000	1/700,000	1/1,000,000
p-Rosaniline	$-NH_2$, $-NH_2$ $-NH_2$	−	−	+	+	+	+	+	+
Fuchsin	$-NH_2$, $-NH_2$ $-NH_2$, $-CH_3$	−	−	+	+	+	+	+	+
Methyl Violet	$-N(CH_3)_2$ $-N(CH_3)_2$ $-NH\cdot CH_3$	−	−	−	−	−	+	+	+
Crystal Violet	$-N(CH_3)_2$ $-N(CH_3)_2$ $-N(CH_3)_2$	−	−	−	−	−	−	+	+
Malachite Green	$-N(CH_3)_2$ $-N(CH_3)_2$	−	−	−	−	−	−	−	+
Brilliant Green	$-N(C_2H_5)_2$ $-N(C_2H_5)_2$	−	−	−	−	−	−	−	+
Methyl Green	$-N(CH_3)_2$ $-N(CH_3)_2$ $-N(CH_3)_2Cl$	+	+	+	+	+	+	+	+
Soluble Blue	3(—NH⟨⟩SO_3H)	−	−	+	+	+	+	+	+

+ = growth from inoculum of 10^6 cells/ml.
− = no growth.

cells. The effect is not due to any alteration of the assimilation process which has the same properties as before; nor is it due to micelles of dye taking glutamic acid mechanically into the cell since exactly

the same results are obtained by submitting the cells to the dye and washing them free from excess dye prior to the assimilation experiment. Optimum effects are obtained with an amount of dye which bears the same relation to the quantity of cells used, as the amount of dye necessary to inhibit growth bears to the inoculum in the growth tests. The difference between the levels with and without dye may

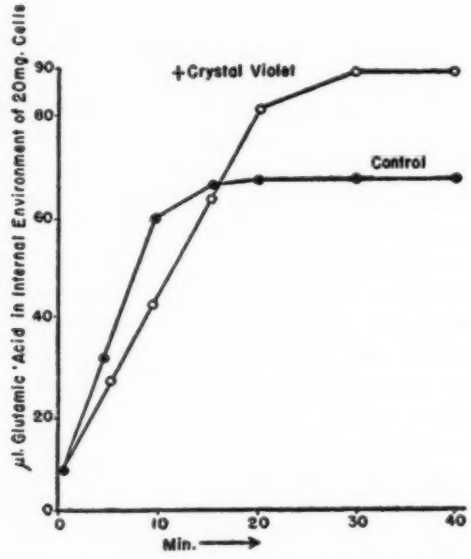


FIG. 1. LECTURE II. Effect of crystal violet (1/10,000) on assimilation of glutamic acid into the internal environment of deficient *Strep. faecalis* cells. Cells incubated at 37° in salt solution containing 0.5% glucose and glutamic acid (200 μl./ml.).

vary widely with the age of the cells; with cells harvested from a 6–8 hr. culture the dye-treated cells may attain an internal level some 200–300% higher than the untreated cells (12).

An increase in the level attained during assimilation should indicate an inhibition of the metabolism of glutamic acid within the cell. This can be checked as follows: deficient cells are suspended in a solution of glutamic acid and glucose and incubated for an hour at 37° as usual,

but the glutamic acid content of the cells and the supernatant is accurately assayed both before and after the incubation. If this is done we find that there is no loss of glutamic acid during the experiment if glucose is not present, i.e. if no assimilation takes place, but that, if glucose is present, then the amount of glutamic acid disappearing from the external environment is markedly greater than that which appears within the cells. In the case quoted, (12), of a total amount of approx. 1800 μl. glutamic acid, 568 μl. disappeared during the assimilation process. It can be presumed that this glutamic acid has been metabolised in such a way that it can no longer be estimated by the decarboxylase method. However if the experiment is carried out in the presence of Crystal Violet and glucose, then the amount of glutamic acid which disappears during the assimilation is little greater than the experimental error.

The results can be checked by taking *Strep. faecalis* cells which contain a large amount of free glutamic acid and suspending them in amino-acid free media; in the absence of glucose no change occurs; in the presence of glucose, glutamic acid passes out of the cell as described before but assays on (cells + supernatant) show that there is a disappearance, in the case quoted (12), of 107 μl. of a total of 250 μl. during the migration. Again if the experiment is repeated in the presence of Crystal Violet, the migration still occurs in the presence of glucose but the amount disappearing from the internal environment is equal, within experimental error, to that appearing in the external medium.

It seems fairly definite that there is a metabolism of glutamic acid within the cell and that this is inhibited by Crystal Violet with the result that there is a rise of free glutamic acid within the cell.

You will remember that the triphenylmethane dyes vary in anti-bacterial activity with structure; we can determine their comparative effects on this metabolism by studying their effects on the level achieved during assimilation. Table 2 summarises various properties of the dyes using Crystal Violet as the standard for comparison. It can be seen clearly that the effect on metabolism is closely parallel to the anti-bacterial action as judged by the inhibition of growth, suggesting that the latter is related to the former. It has often been suggested, since the biologically active form is the cation, that the dyes vary in

efficiency due to varying ionisation with structure; it is certainly true that these dyes are all more effective in alkaline than acid media while acidic derivatives have no activity and are not fixed by the bacterial cells. However, it can be seen that there is no correlation between the effect on the intact cell and the ionisation constant, and that the most basic of all the series, methyl green, is devoid of activity

TABLE 2 (LECTURE II)

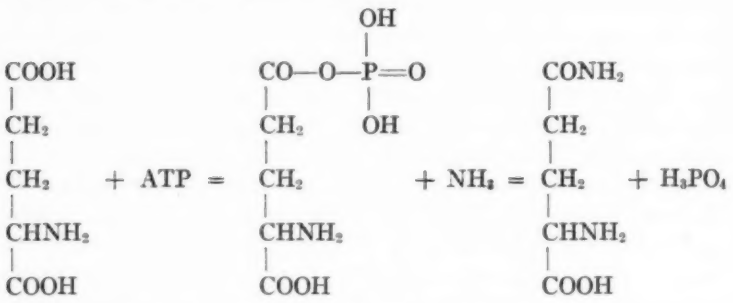
Properties of Triphenylmethane Dyes

NAME	SUBSTITUENT GROUPS	INHIBITION INDEX CV = 100	ASSIMILATION INDEX CV = 100	PARTITION COEFFICIENT ISOBUT./WATER CV = 100	pKa
p-Rosaniline	$-NH_2$, $-NH_2$, $-NH_2$	10	4	16	7.57
Fuchsin	$-NH_2$, $-NH_2$ $-NH_2$, $-CH_3$	10	25	19	
Methyl Violet	$-N(CH_3)_2$ $-N(CH_3)_2$ $-NH\cdot CH_3$	60	85	82	
Crystal Violet	$-N(CH_3)_2$ $-N(CH_3)_2$ $-N(CH_3)_2$	100	100	100	9.36
Malachite Green	$-N(CH_3)_2$ $-N(CH_3)_2$	140	120	164	6.90
Brilliant Green	$-N(C_2H_5)_2$ $-N(C_2H_5)_2$	200	143	632	7.90
Methyl Green	$-N(CH_3)_2$ $-N(CH_3)_2$ $-N(CH_3)_2Cl$	<1	0	0.25	

on growth or metabolism. When we remember that the metabolism is intracellular and that there is a cell-wall which contains a high proportion of lipoid, it seems more probable that the activity of the dyes will depend upon their ability to penetrate the cell-wall. As an indication of the lipoid-solubility of the dyes, their partition-coefficient between isobutanol and water was determined; it can be seen that there is a close correlation between the partition in favour of isobutanol and the biological activity.

The next question which arises is—what is the metabolism of glutamic acid which is inhibited by these dyes within the cell? As we have already seen, we have a wide variety of reactions to test. Many Gram-negative organisms possess a glutamic dehydrogenase which oxidises the substrate to alpha-ketoglutaric acid. The Gram-positive cocci however do not appear to possess this enzyme; if they do its activity is so weak that it comes within the experimental error of the test method. A variety of organisms possess glutamic decarboxylase but this enzyme has not yet been found in either *Strep. faecalis* or *Staph. aureus*. Lichstein & Cohen (1) have demonstrated that these organisms, and many others, possess a highly active transaminase catalysing transamination between glutamic acid and oxalacetic acid; the enzyme can be obtained in a cell-free state from *Strep. faecalis* and Prof. Gunsalus kindly gave us some to test with the triphenylmethane dyes: the enzyme showed little sensitivity to Crystal Violet and significant inhibition was obtained with concentrations of dye 100–1000 times those used in our metabolism tests. It is of course difficult to judge what the inhibitory concentration of the dye within the cell is, but this seems well outside reasonable limits. The growing bacteria must, of course, condense glutamic acid into protein and into peptide structures of the folic acid and glutathione types; if this were the metabolism involved here it should be possible to recover the disappearing glutamic acid by acid hydrolysis. The assimilation experiments with dyes are carried out with washed suspensions and we can find no evidence of protein formation in such preparations. Acid hydrolysis does not lead to a recovery of the "lost" glutamic acid so it is unlikely that protein formation is the reaction inhibited in these experiments. That appeared to be the sum total of testable hypothesese at the time this work was originally carried out and we had to fall back on the old cliché of "metabolic pool" to explain the lost glutamic acid. However during 1947 Elliott (13) in Cambridge, and Speck (14) working independently, discovered that there is another type of glutamic acid metabolism involving phosphorylation. When Krebs (15) was working on the synthesis and breakdown of glutamine in mammalian tissues, he found that synthesis will occur only if energy is supplied by some activity such as glycolysis. Elliott now found that glutamine synthesis will occur if tissue extracts are incubated

with glutamic acid and ammonia in the presence of adenosine-triphosphate and that inorganic phosphate equivalent to the glutamine is liberated during the synthesis. He has isolated the enzyme and it appears probable that the first step in the synthesis is a phosphorylation of glutamic acid in the gamma-position.



In the presence of ammonia an interchange takes place between the phosphate group and ammonia to give glutamine with release of inorganic phosphate. Ammonia is not however the only possible reactant; in the presence of hydroxylamine, the hydroxamic acid is formed and this can be estimated colorimetrically. We have not yet had time to explore the full possibilities of this reaction although it may play a part in the formation of any γ-glutamyl compound. Elliott discovered the enzyme first in brain tissue and we then proceeded to look for its presence in bacteria. The work has not yet progressed very far but we have found a highly active enzyme in *Staph. aureus*. Washed suspensions of the organism have no activity but if the cells are mechanically disintegrated the enzyme is released in an active state. The bacterial enzyme has two interesting properties: first, it is competitively inhibited by methionine sulphoxide and so provides a further example of competitive inhibition by metabolite analogues (16). Methionine sulphoxide was first reported by Waelsch *et al.* (17) to act as a competitive inhibitor of the utilisation of glutamic acid by *L. arabinosus*; since it was ineffective in the presence of glutamine these workers suggested that it inhibited the formation of glutamine from glutamic acid and this suggestion has now been proved with the cell-free system. The formation of the

hydroxamic acid is 72% inhibited by M/90 methionine sulphoxide if the concentration of glutamic acid is M/30 while a four fold increase in the glutamic acid concentration results in a 20% decrease in the inhibition by the same concentration of methionine sulphoxide.

Secondly, the enzyme system is sensitive to Crystal Violet. The activity of the cell-free enzyme system was completely inhibited by a concentration of M/4,000 Crystal Violet (or 1/10,000 dilution) which is the order of concentration necessary to inhibit metabolism in the experiments carried out with washed suspensions. The inhibitory concentration is critical as a further dilution to M/40,000 renders the dye ineffective as inhibitor. It will be of interest to determine the relative inhibitory activities of the various triphenylmethane dyes on this cell-free system but we have not yet been able to do this as practical difficulties arise when the hydroxamic acid reaction is carried out in the presence of some of the dyes and we are awaiting purification of the enzyme before proceeding further with the quantitative aspects of the dye inhibition. We do know that p-Rosaniline is a much less effective inhibitor than Crystal Violet.

We still cannot state categorically that this enzyme which carries out a phosphorylation of glutamic acid is necessarily the metabolic system which is inhibited by the dyes in the intact cells and whose inhibition results in a cessation of growth. All we can say is that this enzyme is one involved in the intracellular metabolism of glutamic acid and is sensitive to Crystal Violet in concentrations which probably approximate to those holding within the cells exposed to the dye; also that none of the other metabolic systems tested that might possibly be involved are sensitive to Crystal Violet in concentrations of this order.

The work just described concerning the effect of dyes on assimilation and metabolism of glutamic acid proves the truth of the suggestion that a rising level inside the cell during assimilation may be due to inhibition of intracellular metabolism. If we study the ability of *Strep. faecalis* cells to assimilate glutamic acid with and without Crystal Violet at various phases of their growth, we get curves such as those shown in fig. 2. The top curve represents the level obtained in 100 mg. cells harvested at various times during growth when allowed to assimilate glutamic acid to saturation in the presence of Crystal

Violet. The experiments are carried out with washed cells and the level obtained must represent the assimilation achieved in the absence of intracellular metabolism. It can be seen then that the rate of assimilation is roughly constant throughout the growth period but falls rapidly after cell-division has ceased. This emphasises the necessity for using cells harvested during the growth period for experiments of this nature. The second curve represents the level obtained

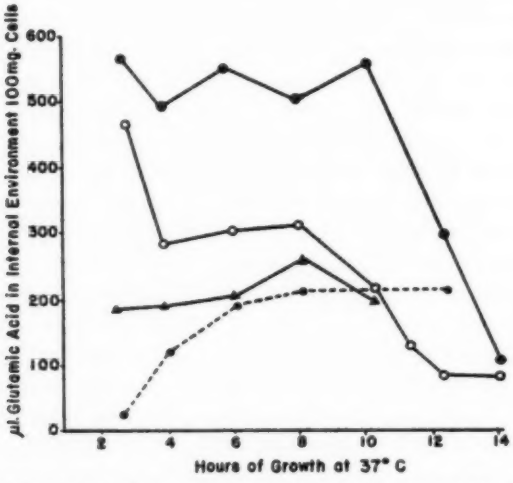


FIG. 2. LECTURE II. Internal concentration of glutamic acid in *Strep. faecalis* cells at various ages of culture. Curve 1 = accumulation within growing cells; 2 = accumulation within resting cells; 3 = accumulation within resting cells treated with crystal violet; Curve 4 = growth curve.

with the cells in the absence of dye; in each case the level is determined by the balance between the rate of assimilation and the rate of metabolism, so the difference between the two curves is a measure of the rate of dye-inhibited metabolism. This appears to be maximal during the linear phase of growth and to fall almost to nil soon after the cessation of cell-growth—as a routine measure we have used 6 hr. cultures for all these experiments.

In these experiments the cells have been grown in a medium containing about 200 μl. free glutamic acid/ml., made into washed sus-

pension and then allowed to reach assimilation equilibrium in a medium containing 200 μl. free glutamic acid/ml. If we estimate the glutamic acid content of the cells directly on harvesting we get curve 1 which meets curve 2 at the time when active cell growth finishes. The lower level in growing cells as compared with washed cells equilibrated with the same external concentration of glutamic acid would lead us to expect that some form of internal metabolism is taking place in the growing cells over and above that in resting cells. Remember-

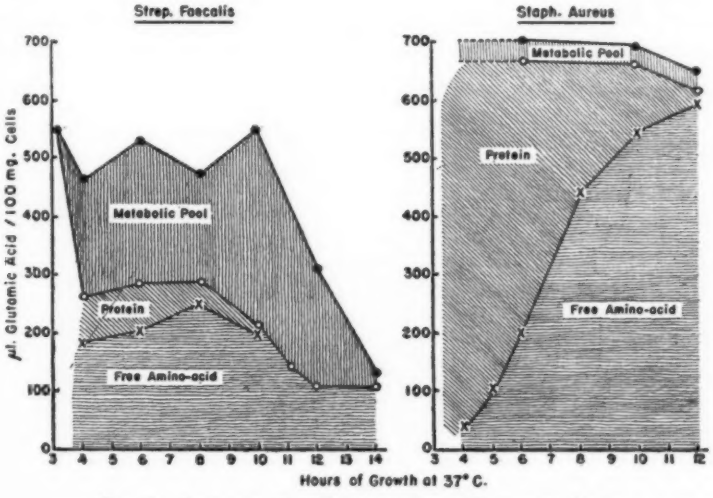


FIG. 3. LECTURE II. UTILISATION OF GLUTAMIC ACID

ing again that the levels indicated in this diagram represent a balance of rates it can be seen that this form of metabolism proceeds at its greatest rate in early cultures and ceases when growth ceases.

Fig. 3 compares the types of curves obtained for *Strep. faecalis* and *Staph. aureus* and it can be seen that there are large quantitative differences between the two organisms. In *Staph. aureus* the rate of metabolism in washed cells is small compared with the rate of assimilation but the metabolism in growing cells is more striking than that in *Strep. faecalis*. I shall show in a minute that this new form of metabolism is protein synthesis and fig. 3 shows that, in *Staph. aureus*, the

rate of protein synthesis is nearly as great as the rate of assimilation in cells harvested during the early stages of growth but that, as growth continues, the rate of protein synthesis falls off in comparison with the rate of assimilation so that free glutamic acid steadily accumulates within the cells. At the end of growth, protein synthesis ceases and nearly all the assimilated glutamic acid appears in the free state within the cell.

The decarboxylase technique estimates only the free form of the amino-acid so, if peptide condensation takes place, the assay of glutamic acid will decrease. Acid hydrolysis will however recover the free amino-acid again and render it estimable by the enzyme. Table 3

TABLE 3 (LECTURE II)

Free and Combined Glutamic acid content of growing Staphylococcus aureus *cells*

AGE OF CULTURE	GROWTH (MG. DRY WEIGHT OF CELLS/ML.)	GLUTAMIC ACID (μl./100 MG. CELLS)			
		Free	Total	Combined	Ratio Combined/Free
hr.					
3	0.07	160	1050	890	5.58
4	0.127	188	981	793	4.23
4½	0.164	215	1083	868	4.04
5	0.179	289	1116	827	2.86
6	0.232	371	1147	776	2.09
7	0.264	405	1134	729	1.80
8½	0.302	405	1003	589	1.48

shows the results of glutamic acid assays on cells before and after acid hydrolysis. When results are expressed in terms of 100 mg. cells, it can be seen that the total glutamic acid content is constant whatever the age of the culture but that the free glutamic acid content rises as the culture approaches the cessation of growth—consequently the combined glutamic acid decreases with increasing age of culture.

Referring back to fig. 3 it can be seen that the difference between curves 2 and 1 can be accounted for in terms of combined glutamic acid. In the growing cell the level of free glutamic acid is lower than that in the resting cell; the difference in the two levels is due to the glutamic acid entering into protein synthesis and can be accounted for by recovery on acid hydrolysis. This means, incidentally, that

the reaction being studied in these curves is a condensation of glutamic acid into protein as glutamic acid and not after transformation into other amino-acids. The changing conditions within the cell are emphasised by the changing ratio of total/free glutamic acid with aging of the culture (18).

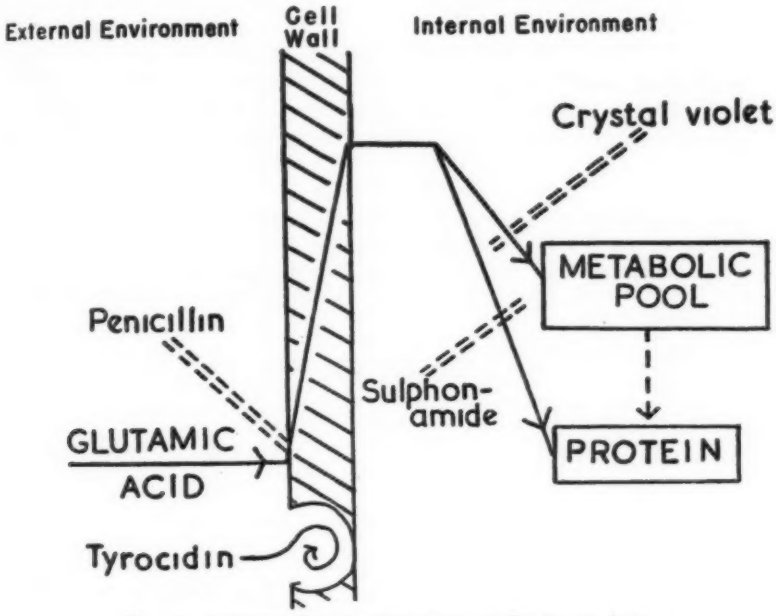


FIG. 4. LECTURE II. ASSIMILATION OF GLUTAMIC ACID

Fig. 4 summarises the position we have reached at this moment. Glutamic acid enters the cell as a result of an active process requiring glycolysis or other exergonic metabolism on the part of the cell to carry the amino-acid into the cell against the concentration gradient. The level within the cell is determined by the balance between the rate at which the amino-acid crosses the cell-wall and the rate at which it is metabolised within the cell. By a study of the effects of growth and of dyes, we have shown that glutamic acid undergoes at least two forms of metabolism within the cell; first, a direct condensa-

tion to protein within the growing cell at a rate which varies with the age of the culture and second, a form of metabolism which is inhibited by dyes of the triphenylmethane series and which probably involves, as the first stage, a phosphorylation reaction. It is obvious that the assimilation process followed by concentration in the internal environment provides the cell with a reservoir of free amino-acid for its anabolic processes.

I want to turn now to the effect of sulphonamides on the utilisation of glutamic acid. Sulphathiazole in saturated solution has no significant effect on the level achieved within the cell during assimilation of glutamic acid by washed suspensions of either *Strep. faecalis* or *Staph. aureus*, nor has it any effect on the glutamine-synthesising enzyme. As many of you will know, it is difficult to demonstrate an action of sulphonamides on Staphylococci growing in peptone or amino-acid media as the peptone and certain amino-acids are antisulphonamide in action. However it is possible to demonstrate a significant slowing of the rate of growth in such media by high concentrations of sulphathiazole. If we add sulphathiazole to cultures of *Staph. aureus* when these are passing out of the lag phase of growth, we get a slowing down of growth and table 4 shows that the glutamic acid level within cells harvested a few hours later is significantly higher than that in cells from comparable cultures without sulphathiazole; further, the magnitude of the increase is related to the concentration of sulphathiazole in the medium. Fig. 5 shows the same effect when samples are taken at

TABLE 4 (LECTURE II)

Effect of Sulphathiazole on accumulation of glutamic acid in internal environment of growing Staphylococcus aureus

All cultures inoculated at time 0 with same strain of *Staph. aureus* and incubated at 37°C. Sulphathiazole added as below at 1 hr., all cells harvested at 4½ hr. and washed before assay.

SULPHATHIAZOLE CONTENT OF GROWTH MEDIUM (MG./100 ML.)	GROWTH AT HARVESTING (MG. DRY WT. CELLS/100 ML.)	GLUTAMIC ACID IN INTERNAL ENVIRONMENT (µL./100 MG. CELLS)
0	0.146	198
1	0.135	280
10	0.121	306
100	0.102	325

various times during the growth period and the rise of internal level in the early stages of growth compared with that in the culture without sulphathiazole can be clearly seen. The rise of level within the cell is, as we know, associated with inhibition of intracellular metabolism. Further we know that the difference between the level of free glutamic acid within the growing cell in early and late cultures, is due to the

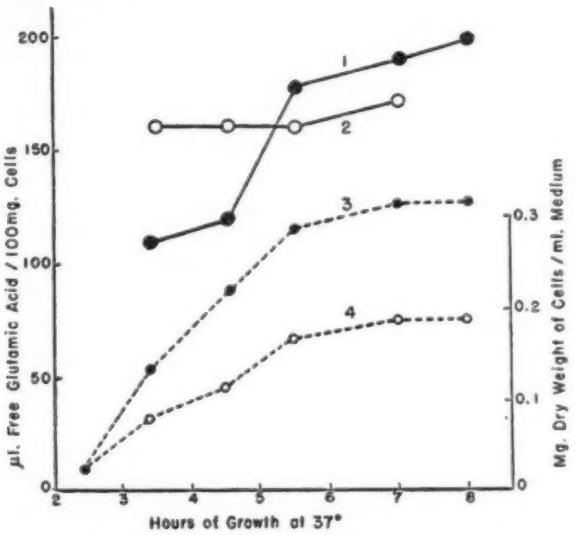


FIG. 5. LECTURE II. EFFECT OF SULPHATHIAZOLE ON ACCUMULATION OF FREE GLUTAMIC ACID IN GROWING STAPH. AUREUS

Curves 1 and 3: Glutamic acid accumulation and growth in absence sulphathiazole. Curves 2 and 4: in presence of 100 mg.% sulphathiazole.

greater rate of protein condensation occurring in the early cultures. It follows then that the effect of sulphathiazole is to inhibit the condensation of glutamic acid into protein and it can be shown that the ratio of total/free glutamic acid in the sulphathiazole-treated cells is much lower than usual for cultures of this age and approximates to that attained in normal cells at the end of growth.

It is not possible to state categorically that sulphathiazole interferes with protein synthesis itself from these results as any reaction which

affected the rate of protein synthesis would have a similar result. I believe it is accepted fairly generally that one primary reaction of sulphonamides is to inhibit the metabolism of p-aminobenzoic acid, as first demonstrated by Woods (19). More recently we have seen that p-aminobenzoic acid forms part of the structure of pteroic acid and the folic acid complex, and Lampen & Jones (20) and also Woods [private communication] have shown that the synthesis of pteroylglutamic acid from p-aminobenzoic acid is competitively inhibited by sulphonamides. It seems probable (21) that p-aminobenzoic acid may play rôles other than that involved as part of the folic acid complex in bacteria and a number of studies (22, 23) have indicated that either p-aminobenzoic acid or folic acid is involved in the synthesis of some amino-acids, methionine in particular. I showed just now that the reaction whose rate we study in these assimilation curves is that of the condensation of glutamic acid into protein; this necessarily involves condensation of other amino-acids so interference with the synthesis of any other amino-acid involved in the protein synthesis would necessarily result in a slowing of the rate of glutamic acid condensation. Consequently these results do not necessarily involve any new mechanism of sulphonamide inhibition but merely demonstrate one of the consequences of such inhibition.

However one of the amino-acids which has been reported to have an antisulphonamide action is glutamic acid and in the course of these studies we received the impression that the higher the glutamic acid content of the cell, the less susceptible to sulphonamide inhibition it became. We confirmed this in the case of a strain which is fairly sensitive to sulphathiazole in the following manner: a nutrient medium was prepared from casein-hydrolysate treated to remove vitamins and which was incubated with glutamic decarboxylase until all the glutamic acid was destroyed; this was then made up with a suitable salt mixture, glucose, nicotinamide and thiamine. Graded amounts of glutamic acid were then added and the growth in the presence of sulphathiazole tested for each concentration. Fig. 6 shows that the presence of glutamic acid increases the sulphathiazole resistance; if we compare the effect of external glutamic acid concentration on internal concentration with its effect on sulphathiazole resistance, we can see that there is a correlation between the two curves which suggests that

the sulphathiazole resistance is determined by the internal concentration of glutamic acid rather than the external concentration.

Fig. 7 shows the accumulation of free glutamic acid within the cells of four strains of *Staph. aureus* growing under identical conditions in the same casein-digest medium. The curves are all of the type with which we are now familiar, showing a decreasing rate of protein synthesis and consequent accumulation of free glutamic acid throughout

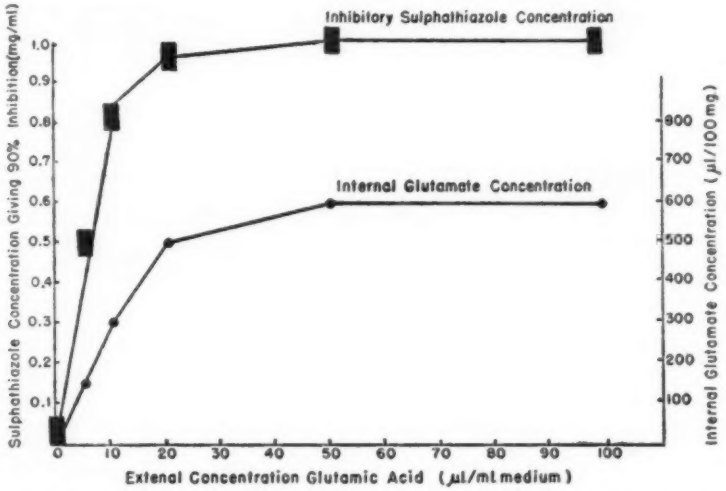


FIG. 6. LECTURE II. Effect of internal concentration of Glutamic acid on (a) internal concentration of glutamic acid and (b) concentration of sulphathiazole necessary to give 90% inhibition of growth.

the growth period. The four organisms differ in their sulphathiazole resistance: Duncan and 156 are resistant to 10 mg.% in growth inhibition tests; 6773 is sensitive to 1 mg.% and the resistance of 1560 is intermediate between the two, somewhere around 5 mg.%. These curves were determined in the hope that they would reveal some difference in the glutamic acid assimilation which could be correlated with the sulphathiazole resistances. It is immediately obvious that it is not the rate of assimilation itself which is involved: a measure of this is obtained by the level at the end of growth when protein synthesis

is over. The rate of protein synthesis is determined by the difference between this final level (strictly speaking, the final level determined in the presence of an inhibitor such as Crystal Violet but the difference does not affect the argument in these cases) and the level at each age of culture; even in young cultures when the rate of protein condensation is greatest, there is no correlation between this rate and

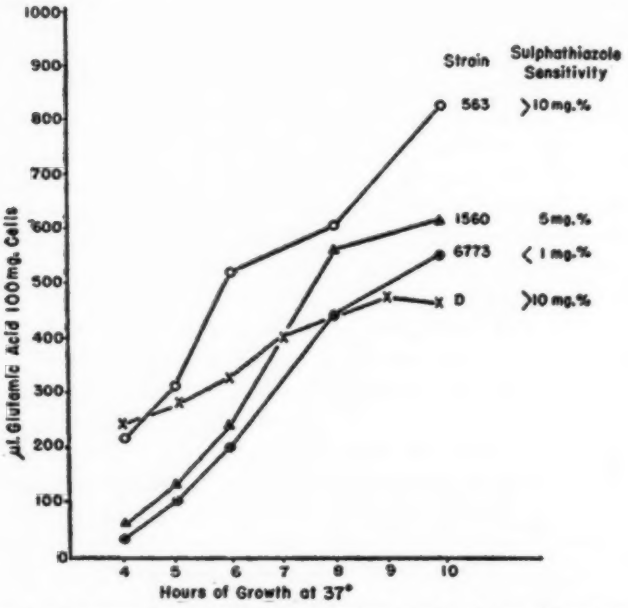


FIG. 7. LECTURE II. Accumulation during growth of free glutamic acid in internal environment of 4 strains of *Staph. aureus* of differing sulphathiazole sensitivity.

sulphathiazole resistance. There is however one quantity which shows correlation with sulphathiazole resistance and that is the height of the level of free glutamic acid in the young cells; the resistant organisms have high levels at about the same value; there is very little free glutamic acid in the most sensitive strain, and the intermediate organism shows an intermediate level. It must again be emphasised that this

level represents the balance of the rate of assimilation over the rates of internal metabolism and also represents the "driving head" of glutamic acid within the cell at this age of culture. As such it will effect by mass action any reaction involving glutamic acid as one of the reactants.

It is tempting to wonder whether it is the synthesis of pteroylglutamic acid again which is involved in the relation between glutamic acid and sulphathiazole resistance. This point is now being investigated but insufficient evidence has been obtained to enable us to make any definite statement.

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LECTURE 3

THE NATURE OF PENICILLIN SENSITIVITY IN STAPHYLOCOCCUS AUREUS

The studies so far described on the assimilation and utilisation of glutamic acid by Gram-positive bacteria may be summarised in this manner. Glutamic acid enters the cell as a result of an active process on the part of the cell and undergoes marked concentration during its passage across the cell-wall. The maximum degree of concentration attainable across the cell-wall varies with strain and species, but the level of free glutamic acid measured within the cell depends upon the balance between the rate at which the amino-acid is assimilated into the cell and the rate at which it is metabolised within the cell. We have been able to identify a number of reactions going on inside the cell and all of these make it clear that the free glutamic acid accumulated within the cell acts as the reservoir upon which the cell draws for its anabolic reactions. The various inhibitors that we have studied in the last lecture had their actions on these intracellular reactions and now, in this last lecture, I wish to turn to an inhibitor which has an action on the assimilation process rather than on the intracellular utilisation.

The story of the discovery of penicillin is too well-known to need recounting, and rates as a monument to scientific international co-operation and as the most beneficial accident ever to happen in a bacteriological laboratory. Penicillin is excreted by the mould *Penicillium notatum* and has very powerful bactericidal action on a variety of bacterial species, mostly belonging to the Gram-positive group although the Gram-negative Neisseria are amongst the most susceptible organisms. Although a vast literature has accumulated concerning the production, chemistry, conditions of action and clinical applications of penicillin, there has been comparatively little published on the mechanism of penicillin inhibition. Chain & Duthie (1) showed that whereas penicillin has no effect on the respiration of resting cells, its addition to growing cultures of *Staph. aureus* gives rise to a progressive inhibition of respiration until this eventually ceases altogether. The fact that penicillin will act only on cells in a growing state was clearly demonstrated by the finding that a bacteriostatic agent such as helvolic acid will protect the cells against penicillin

whereas sulphonamides, which do not exert their action until the cells have undergone several divisions, have no such protective action. These workers also found that if penicillin is added to a growing culture in the lag or logarithmic phase then growth continues to the extent of not more than one division per cell, after which cell-division ceases although increase in cell size without division may then take place. Cessation of growth is followed by a fall in both viable and total counts and general lysis may take place after a period of some hours. Hirsch (2) working independently at the same time found similar effects upon the respiration of *Staph. aureus*—a progressive falling-off in the respiration after penicillin addition to growing cells but not to resting cells—and suggested that the action of penicillin is to produce a degenerative change which results in the production of a sterile generation of cells. Krampitz & Werkman (3) have shown that high concentrations of penicillin will inhibit the dissimilation of ribonucleic acid added to suspensions of *Staph. aureus* and will also inhibit the endogenous breakdown of ribonucleates while having no effect on the metabolism of desoxyribonucleates.

Fig. 1 shows the effect of the addition of various concentrations of penicillin to growing cultures of *Staph. aureus*. If the increase in bacterial substance is followed turbidimetrically we find that growth continues at the normal rate for about 30 mins. after the penicillin addition and the rate then falls off and stops after a time which depends to a certain extent on the concentration of penicillin used. The addition of 10 units penicillin/ml. brings growth to a stop within 90 min. whereas 0.1 units/ml. takes $2\frac{1}{2}$–3 hr. to become completely effective. Since we require a reasonable quantity of organism for estimations of the nature discussed in these lectures we cannot work with organisms harvested during the very early phases of growth and, in general, it has been the custom to add penicillin at about the third hour of growth and harvest at the 4th. or 5th. hour. Fig. 2 shows what happens to the accumulation of glutamic acid within the cells of a culture of *Staph. aureus* grown in a casein-digest medium. In the absence of penicillin, the free glutamic acid content of the cells shows a steady increase throughout the growth period, as shown in the last lecture. If penicillin is added to the culture at the 4th. hour of growth, the accumulation of free glutamic acid continues almost at the normal rate for

approximately an hour and the level then falls dramatically, finally coming to a new steady value at about the time that growth is completely inhibited.

The work we have discussed so far has demonstrated that the level of free glutamic acid within the cell is determined by the balance between

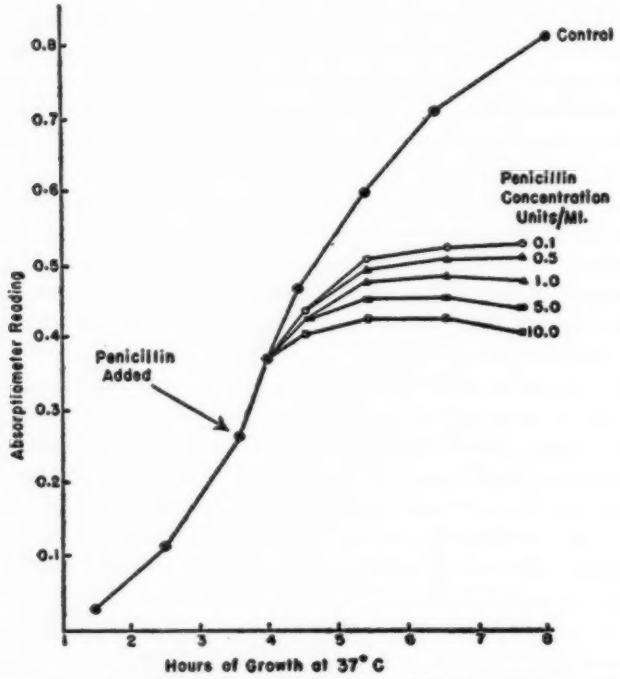

FIG. 1. LECTURE III. EFFECT OF ADDITION OF PENICILLIN TO GROWING CULTURES OF STAPH. AUREUS

the rate of assimilation and the rate of internal metabolism. The addition of penicillin to the culture is followed by a rapid falling of the level within the cells; in agreement with our hypothesis it should therefore follow that penicillin either promotes an increase in the rate of internal metabolism or, alternatively, produces an inhibition of the assimilation process. It is possible to investigate the latter suggestion

by the method outlined in the first lecture of this series: if the organism is grown in a deficient medium, then incubated in a salt medium containing a comparatively high concentration of glutamic acid and

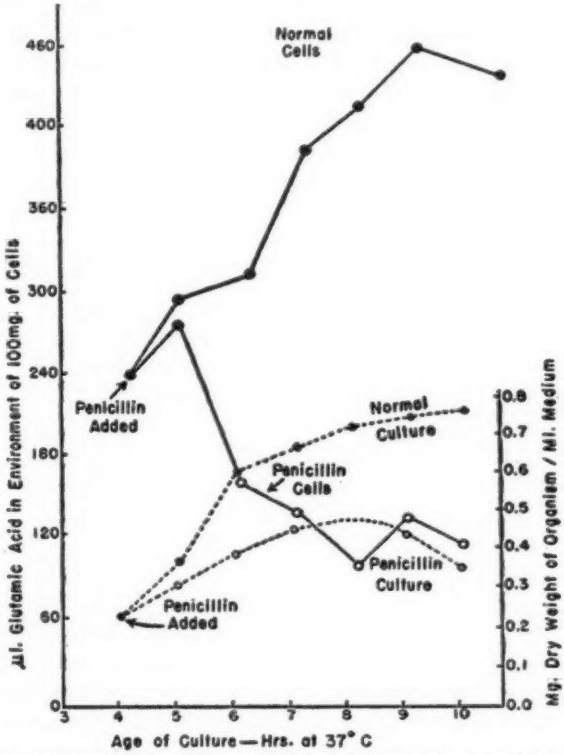

FIG. 2. LECTURE III. Effect of addition of penicillin to growing culture of *Staph. aureus* on the internal accumulation of glutamic acid.

glucose to provide energy for assimilation, the cells will take up glutamic acid until the new equilibrium between internal and external environments is established. The rise in the internal level under standard conditions of time and external invironment can then be taken as a measure of the assimilation ability of the cells. Table 1

shows the amount of glutamic acid assimilated by 100 mg. cells grown under various conditions and tested in this way. Penicillin was added to the culture at the 4th. hour as usual and portions of the culture—together with similar portions from a control culture without penicillin—removed at intervals after the addition. In the control culture, the ability to assimilate glutamic acid is approximately constant (between 600 and 700 μl/100 mg.) over the period examined. In the culture to which 10 units penicillin/ml. has been added, the ability to assimilate is reduced to 14% of the control within 30 min., to 4% within 1 hr. and after 1½ hr. assimilation is no longer possible. Smaller concentrations of penicillin have the same effect of blocking assimilation but take longer to become completely effective. If we compare the impairment of assimilation with the impairment of growth, we can see that there is a close correlation between the two effects (4).

TABLE 1 (LECTURE III)

Effect of the presence of penicillin during growth on the assimilation of glutamic acid by Staphylococcus aureus

Cells grown in a deficient medium and penicillin added in all cases after 3½ hr. growth at 37°C. Cells harvested at various times after the penicillin addition, incubated for 1 hr. at 37°C. in glutamic acid (200 μl./ml.) and glucose (0.5%) and the increase in the internal glutamic acid content (expressed as per 100 mg. cells) determined.

PENICILLIN CONCENTRATION (UNITS/ML. MEDIUM)	GLUTAMIC ACID ASSIMILATED/100 MG. CELLS				
	Time of harvesting after penicillin addition:				
	30 min.	1 hr.	1½ hr.	2 hr.	3 hr.
0	561	702	602	590	614
0.1	—	—	—	130	—
0.5	—	—	—	113	—
1.0	—	—	87	—	0
5.0	—	—	0	—	0
10.0	82	31	0	0	0

It is important to discover to what extent this effect on assimilation is symptomatic of a general metabolic impairment. Table 2 shows the general metabolic activities of cells harvested from a culture which has grown in the presence of 10 units penicillin/ml. for 90 min. when compared with the activities of cells from a control culture without penicillin. The respiration is slightly below normal but the difference

is within experimental error—consequently the respiratory failure demonstrated by Chain & Duthie, and by Hirsch, has not yet set in. The oxidation of glucose and its fermentation are normal, consequently the impairment of assimilation is not a secondary effect due to impairment of the energy system. The assimilation of lysine is normal; you will remember that this process differs from the assimilation of glutamic acid in that it appears to be a purely physical process, the performance of which depends upon the integrity of the cell-wall; this result therefore demonstrates that no significant degree of lysis can have occurred in the cells at this stage. The assimilation of glutamic acid is completely blocked and the viable count is about 2%

TABLE 2 (LECTURE III)

Metabolic activities of normal Staphylococcus aureus *cells and of cells grown in presence of penicillin*

"Penicillin cells" grown for 90 min. in medium containing 10 units penicillin/ml.

	NORMAL CELLS	"PENICILLIN CELLS"
Respiration Q_{O_2}	21.5	19.6
Glucose oxidation Q_{O_2}	86.5	84.5
Glucose fermentation QCO_2 acid	96	108
Lysine assimilation (μl./100 mg.)	90	96
Glutamic assimilation (μl./100 mg.)	602	0
Comparative viable count	452	9

that of the control. It is important to notice that none of the reactions discussed, including glutamate assimilation, are affected in washed cells by the addition of penicillin even in concentrations of the order of 50 units/ml. The blocking of glutamic assimilation occurs only if the cells are grown for a short period in the presence of penicillin and so can again be correlated with the known antibiotic effects of penicillin on growing but not resting cells.

We now know of four effects produced by penicillin on growing cells: loss of viability; loss of the ability to assimilate glutamic acid; progressive respiratory failure; lysis. The results discussed so far show that assimilatory impairment precedes respiratory failure and lysis but it is not possible to state categorically whether it precedes loss of viability. The curves (fig. 2) showing the falling in glutamic level

after penicillin addition and the effect on growth as judged turbidimetrically show clearly that the level within the cell begins to fall before increase in cell-mass ceases. Further than that it is not safe to go at present.

We know from previous work that the glutamic acid within the cell acts as a reservoir for various forms of metabolism within the cell; it is important for us to know whether this internal metabolism is affected by penicillin. We can investigate this by a modification of the method used to show the inhibition of internal metabolism by dyes of the triphenylmethane series. Table 3 shows the results of such an experiment: normal, "deficient" *Staph. aureus* cells are in-

TABLE 3 (LECTURE III)

Effect of penicillin treatment on internal metabolism of glutamic acid in Staphylococcus aureus

	EXTERNAL ENVIRONMENT	INTERNAL ENVIRONMENT	CHANGE IN EXTERNAL ENVIRONMENT	CHANGE IN INTERNAL ENVIRONMENT	µl. GLUTAMIC ACID METABOLISED
Normal cells					
Initial.....	1586	690	−831	+370	461
Final.....	755	1060			
Penicillin-treated cells					
Initial.....	1646	579	−106	−263	363
Final.....	1540	315			

cubated in a known concentration of glutamic acid in the presence of glucose, and the change in the internal and external glutamic acid assayed after an hour; glutamic acid has left the external environment and passed into the cell with a consequent increase in the internal level, but a total of 461 µl. has been metabolised during the assimilation. An exactly similar experiment was carried out with cells of the same age but harvested from a culture to which 10 units penicillin/ml. had been added 90 min. before harvesting. We find that, in this case, 363 µl. glutamic acid have disappeared in the course of the incubation but a glance at the distribution between internal and external environments of this loss shows a very different picture from that obtained with the normal cells: in this case, very little glutamic acid has disappeared from the external environment—in comparison with the

831 μl. which disappeared in the control—and the metabolised glutamic acid has been withdrawn mainly from the internal environment. In other words, the internal metabolism of glutamic acid has proceeded normally in the penicillin-treated cells and has proceeded at the expense of the internal reservoir whereas, in the normal cells, any such internal utilisation is made good by assimilation from the external environment. In the penicillin-treated cells this is impossible since the passage of glutamic acid across the cell-wall is blocked.

The studies discussed so far were all carried out on a normal penicillin-sensitive strain of *Staph. aureus*. The next question that arises is: what happens with the so-called resistant strains? As you know it is a comparatively easy matter to produce penicillin-resistant strains from sensitive ones by a process of serial subcultivation in media containing steadily increasing concentrations of penicillin. The acquirement of resistance was studied by Demerec (5) who concluded that in every culture there occur mutants which have a somewhat greater resistance to penicillin than the bulk of the cells; when growth occurs in a concentration of penicillin which limits the growh of the bulk of the cells, then selective growth of the more resistant mutants takes place. The "training" process thus consists in gradual selection of more and more-resistant mutants and is presumably only limited by the ability of the organism to mutate in this sense. We took our strain of *Staph. aureus* and proceeded to train it to penicillin in the usual way. When we had reached a resistance of 60 units/ml. (an increase in resistance of some 600 times over that of the organism originally studied) we carried out tests on the blocking of glutamic assimilation in the same way; in this case the assimilation was unaffected by the addition to the medium of 1 or 10 units penicillin/ml. but was completely blocked by the addition of 100 units/ml. Consequently an increase in the growth-resistance to penicillin was accompanied by an increase in the resistance of assimilation processes.

It seemed probable that some property of the assimilation process could be related to the penicillin resistance. Since we wished to study the assimilation process itself in the absence of complications due to internal metabolism, it was necessary to abolish this internal metabolism; to do this we took washed suspensions of the cells (in which we have previously shown that protein synthesis does not take place)

and treated them with amounts of Crystal Violet just sufficient to inhibit the residual internal metabolism. In these cells glutamic acid enters the cell until the internal concentration is in true equilibrium with the external concentration. Table 4 shows that the penicillin resistance of a number of various organisms cannot be correlated with their internal level of glutamic acid under standard conditions and in the absence of internal metabolism; the resistance cannot

TABLE 4 (LECTURE III)

Penicillin resistance and internal concentration of glutamic acid in a series of organisms

ORGANISM	STRAIN	PENICILLIN TEST		INTERNAL CONCENTRATION OF GLUTAMIC ACID μl./100 MG.
		Growth Units/m.	No Growth Units/ml.	
Staph. aureus	563	0.02	0.04	1165
B. subtilis	St.	0.04	0.06	26
Staph. aureus	209	0.05	0.10	560
Staph. aureus	D	0.06	0.08	660
Staph. aureus	6773	5.0	7.0	890
Strep. faecalis	ST	6.0	8.0	534
Staph. aureus	6773	9.0	15.0	880
		15	20	825
		60	70	750
		250	300	740
		2000	4000	705
		6000	—	0

then be correlated with the concentration gradient across the cell-wall (6).

Fig. 3 shows the effect of the external glutamic acid concentration on the internal concentration in the case of two organisms. The curves are somewhat different from those I showed in the first lecture and in which no attempt was made to control the internal metabolism—in the absence of internal metabolism the curves are sharper and flatter. In both cases the internal level is independent of the external concentration except for low values of the latter but it can be seen that the

concentration affected by *Staph. aureus* at very low external levels is considerably greater than that effected by *Strep. faecalis*—in other words, the slope of the *Staph. aureus* curve is steeper than the slope for *Strep. faecalis*. The slopes of these curves represent the affinity of the cells for the assimilation of glutamic acid and, in analogy with enzyme-substrate curves, we can take, as a measure of this affinity, the reciprocal of the external concentration required to produce an

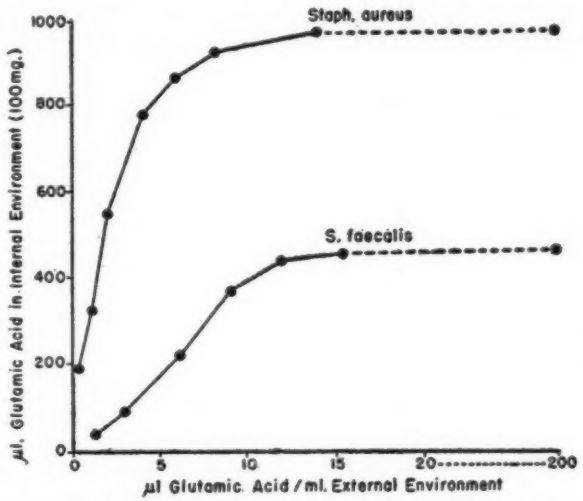

FIG. 3. LECTURE III. DEPENDENCE OF INTERNAL GLUTAMATE CONCENTRATION ON EXTERNAL CONCENTRATION

internal concentration equal to half that attained at saturation. In the case of *Staph. aureus*, this external concentration is 1–2 µl. while for *Strep. faecalis* it is 6–7 µl. *Strep. faecalis* is markedly more resistant to penicillin than *Staph. aureus* and these curves gave us the idea of investigating the value of the "assimilation affinity" in organisms of differing penicillin resistance. Fig. 4 shows a series of such results—the ordinates are expressed as % saturation of the internal environment so as to avoid difficulties due to the varying saturation level in various organisms. Of a series of organisms which had different penicillin-resistances on isolation, there is a correlation in

that the higher the resistance, the higher the assimilation constant (or the lower the assimilation affinity). The correlation was proved by taking the organism with resistance of 15 units/ml. and training it by serial subcultivation in increasing concentrations of penicillin until it would eventually grow in 2000 units/ml. At intervals during the process the assimilation affinity was determined and the curves show that this steadily decreased as the resistance increased.

Fig. 5 shows again the relation between values of the assimilation constant and the log. of the penicillin resistance. Values for a moderately resistant *Strep. faecalis* and for a sensitive *B. subtilis* are in-

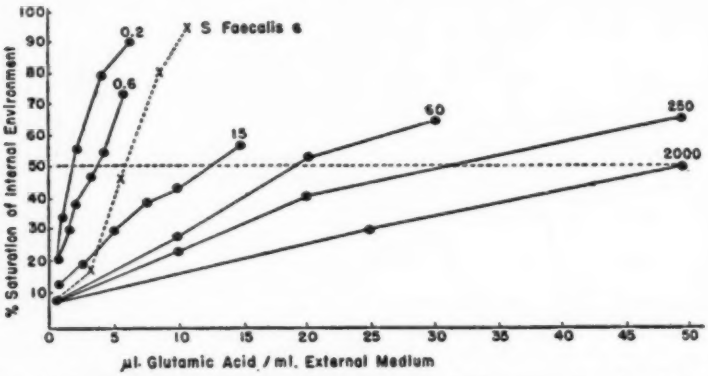

FIG. 4. LECTURE III. "ASSIMILATION AFFINITIES" (GLUTAMIC ACID) OF STAPH. AUREUS OF DIFFERING PENICILLIN SENSITIVITIES

cluded and appear to fit in the general curve given by *Staph. aureus* strains. As the resistance reaches high levels—of the order of 1000 units/ml.—the assimilation affinity decreases very rapidly and it would appear that, if the penicillin resistance were pushed to any further heights, then the organism would no longer be able to assimilate glutamic acid effectively. As the organism is trained to increasing levels of penicillin resistance, the selection of resistant mutants involves also the selection of those mutants which depend less and less upon assimilation for their growth. We have already postulated that assimilation is a mechanism used by the organism to compensate for loss of synthetic power; if assimilation is impaired and the organism

still grows, it would seem from our postulate that it must do so by synthesis.

The organism cultured from 2000 units/ml. appears to be a normal *Staph. aureus* from cultural and staining characteristics. Between

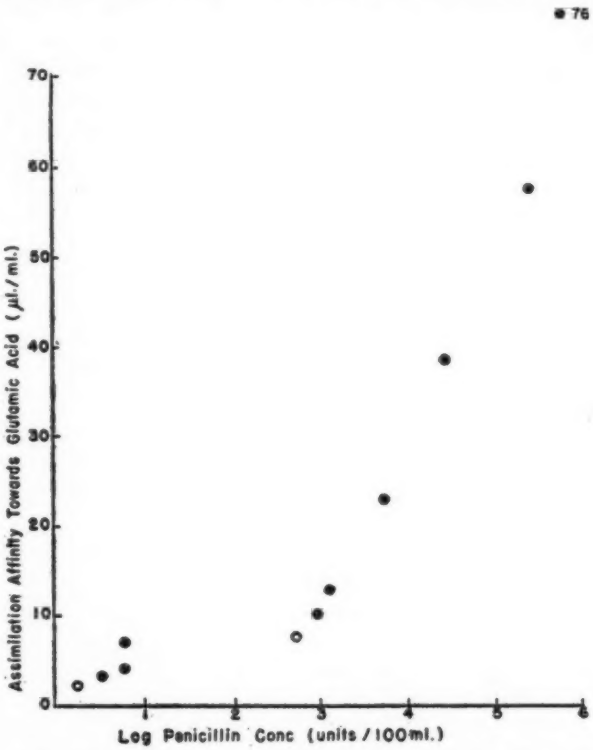

FIG. 5. LECTURE III. RELATION BETWEEN ASSIMILATION AFFINITY TOWARD GLUTAMIC ACID AND THE PENICILLIN RESISTANCE

2000 and 6000 units penicillin/ml. it changes from a Gram-positive coccal organism to a Gram-negative bacillus displaying marked pleomorphism. This spectacular change was first noted by Bellamy & Klimek (8) who were working independently on the penicillin resistance problem at the same time as we were carrying out our as-

similation studies. When their first note was published, I had taken my own organism to a level of 2000 units/ml. and had stopped since I had obtained the information on assimilation affinity just described. When I read the note of Bellamy & Klimek (7) I decided to force my organism, if possible, up to levels reported by these workers to see whether I could get the same changes as they described. My organism was reluctant to increase its resistance above 2000 units, but by perseverance and large inocula it eventually grew first at 4000 and then at 6000 units/ml. Plating of the 4000 unit culture gave a mixture of Gram-positive and Gram-negative forms while the 6000 units culture gave a pure culture of Gram-negative forms. Dr. Bellamy kindly sent me cultures of his resistant organism and I found that it had staining and cultural characteristics identical with mine—obtained from different parent strains, in different media and with different penicillin preparations. Bellamy & Klimek (8) have published details of their training procedure and of the properties of their resistant organism—these properties are duplicated in the case of my resistant organism. Both organisms are strict aerobes and have lost most of their fermentative powers with the exception of a feeble activity towards glucose. Bellamy and I entered into an agreement for the study of these organisms in which I undertook to investigate their amino-acid metabolism.

The general activities of all the organisms are all very similar and are essentially the same as those described by Hills (9) for *Staph. aureus*. In some cases, e.g. glycine, serine, proline and glutamic acid, the rates of attack by the resistant organisms are significantly lower than for the parent strains while in other cases, e.g. arginine and lysine, the rate of attack by the resistant strains is significantly higher. The only case where the rate of deamination is significantly higher than the rate of oxidation is that of arginine; for all four organisms an anaerobic deamination of arginine with liberation of CO_2 can be demonstrated and it is highly probable that all four organisms possess arginine dihydrolase demonstrated by Hills (9) as the main ammonia-forming enzyme system of Gram-positive cocci. All four organisms possess urease to much the same degree of activity. A glance at the figures leaves little doubt but that we are dealing with genuine staphylococcal organisms and not with odd contaminants. This is proved, as we

shall see later, by the fact that the resistant organisms will revert to give Gram-positive *Staph. aureus*.

If the organisms are subjected to the usual assimilation procedure, they show no internal glutamic acid at all under any condition of test. Here they conform to Taylor's law (10) that Gram-positive organisms contain free amino-acids in the internal environment whereas Gram-negative organisms do not. What about their abilities to synthesise amino acids?

Staph. aureus strains are highly exacting towards amino-acids as demonstrated by Gladstone (11) and also require nicotinic acid and thiamine. We investigated the nutritional requirements of the various organisms by growing them first in Gladstone's synthetic medium and then testing their ability to grow in this medium from which the various components were omitted one at a time (12). Table 5 shows the results: the parent 6773 strain requires 10 amino-acids in addition to the two growth factors; the parent 209 requires 7. In the case of both resistant organisms the omission of any amino-acid has no appreciable effect on the growth; likewise the omission of nicotinic acid has no depressant effect. We made a jump and tested the growth of the organisms in a simple salt, glucose, thiamine and ammonia medium; neither parent strain will grow but either resistant organism will grow normally after a lag of a few hours—this lag being considerably reduced by the addition of cystine to the medium. In other words, the two resistant organisms can synthesise all their amino-acid requirements from ammonia and glucose and can also synthesise nicotinamide but not thiamine. The studies which we carried out on assimilation affinity suggested that increased penicillin resistance should select mutants which relied on synthesis rather than assimilation processes; pushed to its logical conclusion this should mean that penicillin training would eventually lead to the selection of mutants which can synthesise all their amino-acid requirements; this has now been realised experimentally.

We have studied the ability to synthesise amino-acids of organisms which represent opposite ends of the scale of sensitivity to penicillin. Can we determine any facts about the intermediate cases or obtain any further information on the relation between synthetic ability and penicillin resistance? Table 5 brings out a number of points bearing on

this problem. Our parent sensitive strains 6773 and 209 are exacting to a variety of amino-acids; the Gram-negative derived organisms can synthesise all their amino-acid requirements and nicotinamide. The case of the organism trained to a resistance of 2000 units penicillin/ml. was the first studied; this organism is Gram-positive, a facultative

TABLE 5 (LECTURE III)

Relation between Penicillin Resistance and Synthetic Abilities

PARENT	MUTANTS SELECTED BY PENICILLIN TRAINING						
	6773	6773	6773	209	209 (PT)	209	209
Penicillin resistance (36 hr. test)	5	2000	6000	0.05	6000	400* (reversion mutants)	1000*
Gram stain	+	+	−	+	−	+	+
Nicotinamide	+	+	−	+	−	−	−
Thiamine	+	+	+	+	+	+	+
Proline	(88)	(88)	−	+	−	(88)	(64)
Histidine	+	+	−	+	−	(64)	(64)
Valine	+	+	−	+	−	(88)	(88)
Glycine	+	(88)	−	+	−	(160)	+
Aspartic acid	+	(64)	−	+	−	(160)	(64)
Leucine	(40)	(40)	−	+	−	(40)	(40)
Cystine	+	+	−	+	−	+	+
Glutamic acid	(40)	−	−	−	−	(64)	(40)
Phenylalanine	(40)	(64)	−	−	−	(40)	(40)
Arginine	+	+	−	−	−	+	+

* Penicillin resistance increased as adaptation took place; after 84 hr. resistance had increased to 800 and 1700 units/ml. respect.

\+ = presence essential for growth.

− = presence not essential for growth in same time as in complete medium.

(88) = growth took place in absence after adaptation had occurred in 88 hr.

anaerobe and normally pigmented; its synthetic abilities fall in a position intermediate between that of the parent 6773 and that of the Gram-negative derivative, in that it adapts to the absence of either glycine or aspartic acid whereas the parent 6773 cannot adapt to the absence of either; also it is non-exacting towards glutamic acid whereas the parent cannot grow in the absence of this amino-acid until adaptation has occurred after 40 hr.

Gladstone (11) showed that strains of *Staph. aureus* can be trained to dispense with amino-acids by the process of attrition involved in serial subcultivation in media containing progressively fewer amino-acids. We have applied the Gladstone technique to both our parent strains and have been able to train them to dispense with all amino-acids except histidine and cystine. Table 6 shows that this training process—which presumably involves selection of non-exacting mutants—results in a simultaneous increase in the penicillin resistance. Con-

TABLE 6 (LECTURE III)

Relation between Synthetic Abilities and Penicillin Resistance

	MUTANTS SELECTED BY NUTRITIONAL TRAINING			
Parent strain	6773	6773	209	209
Nutritional requirements:				
Nicotinamide	+	+	+	+
Thiamine	+	+	+	+
Proline	+	−	+	−
Histidine	+	+	+	+
Valine	+	−	+	−
Glycine	+	−	+	−
Aspartic acid	+	−	+	−
Leucine	+	−	+	−
Cystine	+	+	+	+
Glutamic acid	+	−	−	−
Phenylalanine	+	−	−	−
Arginine	+	−	−	−
Penicillin resistance (36 hr. test) units/ml.	5.0	100	0.05	250

sequently there is a definite correlation between synthetic abilities and penicillin resistance in these strains.

Thirdly we have the interesting cases of two organisms obtained by reversion of the Gram-negative 209 derivative. While Bellamy and his co-workers were attempting to revert their organism to prove its staphylococcal nature, it several times underwent such reversion in our hands—as it were by accident! Between us we have worked out a technique which has enabled us to isolate several Gram-positive staphylococcal reverted mutants from the Gram-negative 209 PT organism. The reversion is due to chance mutation and our methods merely consist in a method whereby the growth of the reverted mutant

will be favoured once the reversion has occurred. The method consists in inoculating large inocula of Gram-negative organisms into a rich casein-digest medium and then two or three subcultivations into a medium containing either marmite or yeast extract in low concencentration as sole nutrient. Table 5 shows the synthetic abilities of two such reversions from 209 PT; both reversions require a number of amino-acids although their requirements are less than those of the original parent 209. One very interesting fact is that both reversions have retained the power to synthesise nicotinamide whereas the original parent—together with most freshly isolated *Staph. aureus* strains—has not this ability. The resistance towards penicillin is again of a high level but intermediate between that of 209 PT and 209. The strains are not stable in their requirements as they adapt fairly easily to the absence of most of the amino-acids—but not of arginine or cystine—and their resistance to penicillin also goes up from about 450 units/ml. in a 30 hour incubation to over 1000 units in a 4 day incubation.

The results expressed in this table bring out two facts quite clearly: first, that *Staph. aureus* possesses a high rate of mutation and second, that there is a reciprocal relationship between the synthetic ability and the penicillin sensitivity of the strains obtained.

This all correlates very well with the original finding that penicillin blocks the assimilation process. We were restricted in our studies on assimilation to glutamic acid although it was always possible that the facts discovered with that amino-acid were symptomatic of changes in the assimilation process towards amino-acids in general. It is now certain that this must be the case; the penicillin-resistant organisms synthesise all their amino-acids and even moderately sensitive strains can dispense with glutamic acid. It is however definite that penicillin does not block the assimilation of lysine, which is, as you may remember, a physical process. Lysine is however not an essential amino-acid for the strains of *Staph. aureus* studied.

We have seen that bacteria must grow either by assimilation or by synthesis of their constituent amino-acids; that some organisms have lost certain synthetic abilities and so are forced to assimilate; that many such obligate-assimilators effect a concentration of certain essential amino-acids within the internal environment prior to further metabolism and that all organisms which have acquired this ability

are also Gram-positive. We have seen that penicillin will block the assimilation process towards certain amino-acids; that training to penicillin-resistance results in the selection of less exacting-mutants; that there is a reciprocal relation between synthetic abilities and penicillin sensitivity; that penicillin-training pushed to its highest levels results in the selection of organisms non-exacting towards amino-acids and that such organisms are Gram-negative; that reversion mutations will occur to give organisms exacting towards amino-acids which are Gram-positive.

The Gram-positive cell is able to effect a concentration of certain amino-acids within the internal environment; a glance at the analytical figures at our disposal shows that the relative concentrations of free amino-acids within the cell are very roughly those of the relative proportions of the combined amino-acids in the cell-protein. It seems possible that the Gram-positive cell represents an evolution from the Gram-negative form such that the evolved form compensates for the loss of synthetic abilities by this concentration of amino-acids within the internal environment in roughly the right proportions for anabolic purposes. There is little doubt but that Gram-negative cells can assimilate amino-acids—the nutrition of *Eberthella typhosa*, *Shigella* and the *Neisseria* proves that,—but they cannot effect the internal concentration. The substance responsible for the positive Gram reaction, the Gram complex, has been studied by Henry & Stacey (13, 14) in England and by Umbreit & Bartholomew (15) in this country. It appears to be a protein with a prosthetic group consisting of Mg ribonucleotide and appears to reside mainly in the surface layers of the cell. If this substance is intimately bound up in the assimilation-and-concentration process, then we may have an explanation of the effect recently shown by Frieden & Frazier (16) of magnesium in increasing penicillin sensitivity, since reduction of magnesium would promote the growth of mutants of a less-Gram-positive character which were, consequently, less able to effect assimilation and more able to carry out synthesis.

Penicillin is only effective on growing cells. When the Gram-positive cell grows, it must synthesise the Gram-complex and this requires magnesium and the synthesis of ribonucleic acid. This immediately reminds us of the studies of Krampitz & Werkman (3) on

the effect of penicillin on ribonucleic acid metabolism in *Staph. aureus*. These workers showed that penicillin will inhibit the breakdown of either endogenous or exogenous ribonucleic acid; high concentrations of penicillin were used (of the order 400–2000 units/ml.) but the workers point out that cell concentrations were used which were about 1000 times the concentrations used in growth inhibition tests. This argument may or may not be valid but there is evidence here that penicillin can disorganise ribonucleic acid metabolism. A suggestion which forces itself upon us at this stage is that penicillin interferes with the synthesis of the Gram-complex or otherwise disorganises the nucleotide composition of the cell such that the concentration and assimilation mechanisms break down. Dufrenoy & Pratt (17) have recently shown that when *Staph. aureus* is grown in bacteriostatic concentrations of penicillin, then the cells gradually lose their Gram-positiveness and eventually become Gram-negative. If there is an actual disorganisation of the nucleotide complex, it should be possible to detect some alteration in the ribonucleic acid/desoxyribonucleic acid ratio under the influence of penicillin. Table 7 shows results we have obtained in such an attempt. A normal penicillin-sensitive *Staph. aureus* was grown in casein-digest-glucose medium and 5 units penicillin/ml. added to portions of the culture at the end of the fourth hour of incubation. Organisms were harvested at the time of the penicillin addition and at intervals of 30 and 120 min. after the addition. In all cases the ribonucleic-phosphate and desoxyribonucleic-phosphate were estimated by the method of Schmidt & Tannhauser (18). In the normal culture there is a steady drop in both ribo- and desoxyribonucleic acid content with age of culture, much as described by Malmgren & Heden (19). The addition of penicillin has been followed by a significant decrease in the ribonucleic-P, 6% below the control after 30 min. and 10% after 120 min., and a marked increase in the desoxyribonucleic-P content amounting to 20% greater than the control after 30 min. and 58% after 120 min. The ratio between ribonucleic-P and desoxyribonucleic-P averages 8.7 for the control culture and has fallen to 6.5 for the culture grown in penicillin for 30 min. and to 5.55 for the 120 min. penicillin-culture. There is definite evidence here of a disorganisation of ribonucleic acid metabolism.

If we calculate the total amount of nucleic acids per ml. of culture

we can see that the synthesis of desoxyribonucleic acid is the same in the presence of penicillin as in its absence while the increase in ribonucleic acid is only 10% in the penicillin culture (after 30 min.) compared with an increase of 42% in the normal culture. If the suggestion of Brachet (20) is correct, that ribonucleic acid gives rise to desoxyribonucleic acid, then these results could be interpreted as meaning that penicillin has inhibited the formation of ribonucleic acid but not its transformation into desoxyribonucleic acid. Analyses

TABLE 7 (LECTURE III)

Effect of Penicillin on the Nucleotide Content of Staph. aureus

Staph. aureus grown at 37°C in casein-digest-glucose medium and 5 units penicillin/ml. added to growing cultures as indicated below. All quantities expressed as μg. P/mg. dry weight of cells.

	AGE OF CULTURE:					GRAM-NEGATIVE RESISTANT ORGANISM
	4 hr.	4½ hr.		6 hr.		
Time for which penicillin was present before harvesting	—	—	30 min.	—	120 min.	—
Total P content	32.6	30.2	31.7	27.6	26.2	15.2
Ribonucleic acid P	20.2	18.8	17.7	15.5	13.9	8.38
Desoxyribonucleic acid P	2.30	2.26	2.71	1.58	2.5	3.06
Ratio $\frac{\text{Ribonucleic-P}}{\text{Desoxyribonucleic-P}}$	8.8	8.3	6.5	9.8	5.55	2.7
Dry weight of cells in culture. (mg./ml.)	0.121	0.190	0.154			
Ribonucleic-P/ml. culture	2.45	3.47	2.72			
Desoxyribonucleic-P/ml. culture	0.25	0.43	0.42			

carried out on the highly resistant Gram-negative organism derived from *Staph. aureus* 6773 show that this has the very low ratio of 2.7 while its desoxyribonucleic-P content is higher than that of the Gram-positive organism from which it was derived. The action of penicillin on the Gram-positive organism has thus had the effect of altering its nucleic acid composition in the direction of that of the Gram-negative organism.

It seems probable that we have reached the limit of the knowledge

that studies of assimilation and amino-acid synthesis can give us about the action of penicillin. The present indication is that we must turn our attention to the study of nucleic acid synthesis and metabolism with especial reference to that material laid down in the Gram-positive cell and imparting the positive reaction when treated by the Gram technique. Perhaps, in turn, such studies might throw some light on the part played by this material in the assimilation-concentration mechanism evolved by the Gram-positive bacterium. We have to bear in mind, through all this, that the Neisseria are Gram-negative but highly penicillin-sensitive. They may possibly represent organisms which have not yet acquired the internal concentration mechanism. For the moment we must content ourselves with reflecting that the cytologist says that the Neisseria are "Gram-intermediate" and that they are certainly unable to synthesise certain amino acids, all the Neisseria so far studied being nutritionally exacting towards both cystine and glutamic acid at least.

The studies that I have outlined in these lectures on the assimilation of amino-acids by bacteria have enabled us to gain a certain insight into the nitrogen metabolism of Gram-positive bacteria and they have, at the same time, raised many new problems for us to investigate. It is, however, one of the delights of scientific research that the answer to each problem raises new ones and that, as we pass each stage in our enquiry, we observe that evolution of knowledge which is described, in the words of Spencer, as a "change from a relatively indefinite incoherent homogeneity to a relatively definite coherent heterogeneity."

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SULFHYDRYL COMPOUNDS AND THE SICKLING PHENOMENON

A Preliminary Report[1]

LEWIS THOMAS and CHANDLER A. STETSON, JR.

From the Department of Pediatrics, Johns Hopkins University Medical School, and The Harriet Lane Home for Invalid Children, Johns Hopkins Hospital, Baltimore, Maryland

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The methods which are in current use for the routine laboratory diagnosis of sickle cell disease are based on the widely accepted concept that the sickling phenomenon is brought about by the reduction of hemoglobin in the red cell. Exposure of susceptible cells to high concentrations of carbon dioxide is known to cause rapid sickling (1). Exposure to atmospheres with sufficiently low oxygen tension similarly accelerates sickling (2). The production of prolonged stasis by a tourniquet has been shown to enhance the sickling in samples of venous blood (3). Spontaneous sickling occurs when suspensions of cells are sealed beneath a cover slip and allowed to stand for some hours (4) (5). This latter procedure is probably the one most commonly employed in routine diagnostic laboratories, but it has the disadvantage that a conclusive diagnosis is often impossible until twenty four hours after the test has been set up.

In the present study, the effect of a variety of reducing agents on sickle cells was tested with the purpose of devising a dependable means for the rapid diagnosis of sickle cell disease. It was found that sulfhydryl compounds, notably hydrogen sulfide, BAL (2,3-dimercaptopropanol), and cysteine caused complete sickling within less than a half hour. This report is concerned with a description of these observations, together with a presentation of certain related findings which may have bearing on the underlying mechanism of sickling.

[1] This work was supported by a grant from the Life Insurance Medical Research Fund.

MATERIALS AND METHODS

Varying concentrations of BAL,[2] cysteine hydrochloride,[3] and glutathione,[4] as well as other reagents to be mentioned below, were prepared by dissolving the chemicals in physiological saline and adjusting the pH to neutrality with sodium hydroxide. Hydrogen sulfide was prepared as a saturated solution by bubbling the gas through saline. For the test, a small drop of the reagent was mixed on a slide with a drop of oxalated blood which had been diluted approximately 1 to 5 with saline. The mixture was immediately covered with a glass coverslip and, if prolonged observation was desired, sealed at the edges with vaseline.

Approximately 50 specimens of blood were obtained from patients with known sickle cell disease. As controls, 30 specimens from normal individuals and 6 specimens from patients with other varieties of anemia were tested.

RESULTS

In Table I are summarized the results of testing a sample of blood in the presence of several thiol reagents. These results are illustrative of those obtained in every case of sickle cell disease studied. The most rapid sickling was seen in preparations mixed with hydrogen sulfide; in most instances sickling was clearly evident within five minutes after the mixture was placed on the slide, and the majority of the cells were fully sickled within fifteen minutes. In the presence of BAL and cysteine, sickling usually became apparent in fifteen minutes and was complete within a half hour. Glutathione was considerably less effective, producing satisfactory sickling only after two or three hours. The concentrations of thiol compounds necessary to produce sickling are indicated in Table I. For practical purposes, the most suitable concentrations were found to be as follows: hydrogen sulfide—an approximately saturated solution; cysteine—0.5 molar solution; BAL—0.1 molar solution. It is of interest that in general the concentrations of each substance which produced sickling were also those which gave a positive nitroprusside reaction, and a negative nitro-

[2] Pure BAL supplied through the courtesy of Dr. Harry Eagle.

[3] 1-Cysteine hydrochloride (Merck and Co., Inc., Rahway, N. J.).

[4] Glutathione (Schwarz Laboratories, Inc., New York 17, N. Y.).

prusside reaction always indicated an amount insufficient to cause sickling.

The thiol reagents did not produce sickling or any other change in contour resembling sickling in the red cells of normal individuals.

TABLE I

The Production of Sickling by Sulfhydryl Compounds

REAGENT	CONCENTRATION	TIME OF OBSERVATION (IN MINUTES)					
		5	10	15	30	60	120
H_2S (saturated solution)	1:1	++*	+++	++++	++++	++++	++++
	1:2	+	+++	++++	++++	++++	++++
	1:4	−	++	+++	++++	++++	++++
	1:8	−	+	++	+++	++++	++++
	1:16	−	−	−	+	++	++++
BAL	0.10M	+	++	+++	++++	++++	++++
	0.05M	−	+	++	++++	++++	++++
	0.01M	−	−	+	++	++++	++++
Cysteine	0.50M	−	+	+++	++++	++++	++++
	0.10M	−	−	++	+++	++++	++++
	0.01M	−	−	+	+	++	++
Glutathione	0.25M	−	−	−	−	+	++
	0.05M	−	−	−	−	−	−
NaCl		−	−	−	−	−	−

*+, approximately 5% of cells sickled.
++, approximately 25% of cells sickled.
+++, approximately 75% of cells sickled.
++++, approximately 90–100% of cells sickled.

Blood from three adult negroes with the sickling trait, who gave no history suggesting sickle cell disease, showed the same response to thiol compounds as that from patients with the active disease. However, in two other adults with the sickling trait, the cells exhibited a delay of two hours before responding to hydrogen sulfide or BAL. The possible significance of this delayed response will depend upon the accumulation of additional data with blood from sickle-trait individuals. At the present time, an insufficient number of such persons has been studied.

Oxygen exerted a marked inhibitory effect on the sickling produced by sulfhydryl compounds, and was also capable of reversing the phenomenon. Sickling did not occur in preparations left open to the air, or in ordinary hanging drop preparations. When the coverslip overlying a fully sickled preparation was removed, the sickled cells rapidly and completely reverted to the normal discoid shape.

The effect of reagents known to be thiol antagonists was tested with several samples of sickle cell blood. It was found that spontaneous sickling, as well as thiol-induced sickling, was completely inhibited by small amounts of o-chloromercuribenzoate,[5] iodoacetamide,[5] iodosobenzoate,[5] hydroquinone and sodium maleate. The interpretation of the inhibitory effect on spontaneous sickling is not yet clear. It is possible that these substances may block an —SH mechanism within the cell, but it is also possible that the effect is due to some other less specific chemical trauma to the cell. Further investigations of the effect are in progress.

The thiol reagents caused a pronounced darkening in the color of the blood within a few minutes after mixing. This color change was always seen with concentrations of thiol compounds which were sufficient to produce sickling. It was accompanied by the disappearance of the absorption bands of oxyhemoglobin, visualized with a spectroscope, and by the appearance of the typical absorption spectrum of reduced hemoglobin.

The erythrocyte sedimentation rate was tested in three patients with the active disease, comparing oxygenated blood with other samples of blood which, following oxygenation, were treated with BAL or hydrogen sulfide. It was found in each instance that hydrogen sulfide and BAL caused a marked reduction of the sedimentation rate, which was of the same degree as that caused by carbon dioxide (6) (7). It was also noted that rouleaux formation did not occur in the samples treated with the sulfhydryl reagents, although it was prominent in untreated samples.

COMMENT

The possible significance of the foregoing observations in regard to the mechanism of sickling is the subject of an investigation which is

[5] Obtained through the courtesy of Dr. Leslie Hellerman.

still in progress. The detailed results of this investigation will be reported at a later date.

For practical purposes, the findings provide a new method by which a rapid and reliable diagnosis of sickle cell disease can be made. In our experience, the simplest and most rapidly active agent of the group tested is the saturated solution of hydrogen sulfide. When a drop of this reagent is mixed on a slide with a drop of blood, sickling of a majority of the cells is evident within fifteen minutes and is not infrequently complete in as short a time as five minutes. It has been found that solutions of hydrogen sulfide maintain their activity for five days if kept in the refrigerator in tightly stoppered containers. BAL and cysteine, on the other hand, are highly unstable and must be prepared in fresh solutions each day and kept cold at all times. The activity of any of the reagents can be verified in rough fashion by means of the nitroprusside test.

SUMMARY

Hydrogen sulfide, BAL and cysteine produce rapid and complete sickling when mixed with the blood of a patient with sickle cell disease. Glutathione causes sickling after a longer interval. Sickling produced by sulfhydryl compounds is rapidly reversed by exposure of the sickled cells to air. The sedimentation rate of oxygenated blood from patients with sickle cell disease is markedly diminished by the addition of hydrogen sulfide or BAL. Spontaneous sickling, as well as sickling by thiol reagents, is inhibited by certain agents known to be thiol antagonists.

It is suggested that sulfhydryl compounds may be of value in the laboratory diagnosis of sickle cell disease because of the rapidity with which reliable and reproducible results can be obtained.

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BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of this journal)

Brain and Intelligence. By WARD C. HALSTEAD. 206 pp. $6.00. *University of Chicago Press, Chicago, Ill.*, 1947.

Although *Brain and Intelligence* undoubtedly will not be one of Chancellor Hutchins' 100 books, it is nevertheless a much needed argument for objectivity in the field of psychological and psychiatric testing. The author makes his central theme "the frontal lobes of the brain, long regarded as silent areas, but in reality the organs of civilization—the basis of man's despair and of his hope for the future." Halstead has utilized his opportunities in the neurosurgical clinic at the University of Chicago, where he saw patients of Walker, Bucy, Bailey and others before and after various brain operations. Among these he lists: cerebral lobectomies, military head injuries, closed head injuries, pre- and post-lobotomies—a total of 207 patients. He applied a number of objective tests, 27 "behavioral indicators", described in an appendix of the book. The volume contains an excellent group of diagrams of the brain lesions of the cerebral lobectomies.

The book begins with a review of some of the neurological, psychiatric, and psychological work leading up to the author's concepts and his argument for "biological intelligence". Previous studies are grouped under psychometric, clinical, and neurological intelligence. With considerable hauteur the author makes short shrift of nearly all past work with the exception of that of Lashley and one or two others. "Professor Halstead has combined new instruments and quantitative methods with the techniques of factor analysis [Thurstone and Holzinger] to isolate the basic factors of higher mental processes," says the jacket review. The results of the author's tests are described in chapters entitled the A Factor, the Power Factor P, the Directional Factor D. Then comes a kind of philosophy of the results under the title "Nuclear Structure of the Ego". Part II deals with "Localization of Function in the Brain."

While some readers will doubtless welcome the relegation of the data to a higher level of discussion and its embellishment by the use of such terms as the Field Factor A, the Power Factor P, the Directional Factor D, and the Nuclear Structure of the Ego, the present reviewer is somewhat bewildered by these highfalutin adornments. The state of confusion in our present thinking and testing which Halstead's data might go far toward dispelling is partly reincarnated by his own special brand of a higher psychological philosophy. The tests are dissected into 26 "quantitative indicators" (p. 171), 17 of which bear the name of the author; thus 15 is "Halstead Time-Sense Test (Vision)", 16 is "Halstead Time-Sense Test (Memory)", 17 "Halstead Dynamic Visual Field Test (Central Form)". Since many of these tests have been previously used under other names and are not

sufficiently modified here to warrant a rebirth under a new nomenclature, one is irritated to see work as good as Dr. Halstead's promises to be thus marred.

Like Little Jack Horner eating his Christmas pie, Halstead has stuck in his thumb and pulled out a plum in his emphasis on the necessity for objective testing, and perhaps he can be excused for the "What a fine boy am I!" This Jack Horner attitude pervading the book, howsoever annoying, should not blind us to the need for such an approach, and the author is to be commended for his enthusiastic argument for objectivity and a demonstration of how objective tests may reveal important data.

W. H. G.

Fundamentals of Psychiatry. 4th edition. By EDWARD A. STRECKER. 325 pp. $4.00. *J. B. Lippincott Co., Philadelphia, Pennsylvania,* 1947.

The latest revised edition of this elementary text is likely to enjoy considerable popularity, as have other editions in the past. Among new chapters, the one on military psychiatry contains an excellent summary of psychiatric knowledge gained during World War II, including a critical evaluation of the subject. The section, "Psychosomatic Medicine and Psychiatry", is practically identical with a corresponding chapter in Strecker, Ebaugh and Ewalt, "Practical Clinical Psychiatry," 5th edition, and gives little clarification of the matter. Although the book is dedicated to the practitioner of medicine, he will find it difficult at times to make ready use of its contents, particularly as regards the treatment of mental disorders. The author's inclusion of a classification of psychiatric illness based on "tension" constitutes an interesting attempt to popularize this terminology. Because of Dr. Strecker's appealing style and his awareness of his audience's needs, the book makes easy and good reading and should stimulate the student's interest to tackle the more complex aspects of psychiatry.

E. A.

Headache and Other Head Pain. By HAROLD G. WOLFF. 642 pp. 152 figures. $12.00. *Oxford University Press, 114 Fifth Avenue, New York 11, New York,* 1948.

In this volume Dr. Wolff records the results of 15 years' study of the natural history of headache, by far the commonest and one of the most distressing of human discomforts.

In an introductory chapter the author grapples with the old, unanswered question of the nature of pain and, after 50 pages, 23 figures, and 125 references, returns to the old view that, to the victim, the quale or feeling state is the most important aspect. He then proceeds to a descriptive analysis of the pain—sensitive structures within the cranial cavity; much of this together with data on the localization and nature of the resultant pain emerged from systematic stimulation of these structures during craniotomy.

Confirming and extending the observations of Cushing and others he has demon-

strated that, with the exception of parts of the dura at the base of the brain, sensitivity to pain is limited to venous sinuses and their tributaries, dural arteries and major arteries at the base. Upon this the author derives six basic mechanisms of headache arising within the cranial cavity: (1) Traction on veins passing from brain surface to venous sinuses and displacement of great venous sinuses (2) Traction on middle meningeal arteries (3) Traction on large arteries at base of the brain or their main branches (4) Distention and dilatation of intracranial arteries (5) Inflammation in or about pain—sensitive structures of the head, and (6) Direct pressure by tumors or adjacent tissue on cranial or cervical nerves, containing many pain-afferent fibers. These he then applies to the explanation of various types of headache.

Most exhaustive (140 pages) is the section on the migraine syndrome which, it is concluded, results from distention of cranial arteries. At the same time the somewhat imponderable nature of the syndrome is implied in its description as "a pattern of dysfunction integrated within the nervous system and manifested as mood changes and widespread bodily disturbances of a non-painful and painful nature."

The book sifts critically the published observations and conclusions regarding head pain and adds an enormous amount of pertinent data obtained by relatively simple experimental approaches to simple questions. The results serve by inference to identify the source of many types of headache and to point the way to treatment.

Illustrations are plentiful and lucid. Most welcome are the brief summaries at the ends of the chapters and a final chapter on clinical differentiation.

E. C. A.

Operative Gynecology. 6th edition. By HARRY S. CROSSEN AND ROBERT J. CROSSEN. Illus. 999 pp. $15.00. *C. V. Mosby Company, St. Louis, Missouri*, 1948.

This is the newest edition of a book which, until recently, was one of the very few authoritative books on this particular division of surgery. It is thoroughly illustrated, and many of the additional illustrations have been reprinted from articles and other books. The color drawings contrast sharply with the excellent colored photographs. There has been considerable reorganization and rearrangement of the chapters in line with the recent advances in gynecology. The subject of uterine cancer and the discussions of x-ray and radium treatment are particularly clear and complete. The authors have given long and critical consideration to carcinoma of the vulva, in line with Taussig's studies and recommendations. The chapters on retrodisplacement of the uterus, prolapse of the uterus, and genital fistulae are particularly thorough. The addition of a short historical sketch and the references to the recent literature on each subject are welcome and useful to both the specialist and the general surgeon. Gynecologists will heartily endorse the authors' constant plea for removal of the involuting or involuted uterus and ovaries

when an operation is necessary. The cancer potential of these organs is stressed and certainly there can be little excuse for removing only one ovary in a postmenopausal patient or for leaving the uterus in a forty year old patient who has been rendered sterile or castrated by the operation. Dr. H. S. Brookes, Jr. has again written the chapters on "The Intestinal Tract" and "Anesthesia".

A few minor criticisms can be made. The sulfa drugs and the new antibiotics are given scant attention and little notice is taken of the ways in which such therapy has altered surgical treatment. Sulfasuxidine was not recommended preoperatively or postoperatively in the treatment of rectovaginal fistulae. The cervical biopsy clamp and procedure are declared out of date; the substitute offered (sharp conization) is often necessary, but cannot practicably replace this simple office instrument and its usefulness. In view of the fact that tuberculosis of the uterus is almost always secondary to tuberculosis of the tubes, the advice on cervical amputation for tuberculosis of the cervix and hysterectomy for tuberculosis of the uterus is misleading and confusing. Sodium pentothal has been the greatest recent advance in gynecological anesthesia, yet it is hurriedly and incompletely discussed.

The authors have inserted much new material and the book is a very useful one for gynecological surgeons and surgeons who occasionally do gynecology.

R. B. S.

Psychobiology and Psychiatry. 2nd edition. By WENDELL MUNCIE. Illus. 620 pages. $9.00. *The C. V. Mosby Company, St. Louis*, 1948.

In this revised edition the general plan and orientation of the first edition have been retained. The long section, 184 pages, of historical parallels along national lines has been omitted. Thus shortened, and printed on thinner paper, the book is physically more attractive and easier to handle.

The new section on treatment has been enlarged and includes a discussion of group therapy. The portion dealing with classification presents the revised Army nomenclature, keyed to the concept of reaction patterns. There is a thirty-page section on "Somatization Reactions or Psychosomatic Reactions."

The distinguishing feature of Dr. Muncie's book is the systematic presentation of Meyerian psychobiology and the ergasiologic formulation of clinical syndromes. One of the major difficulties in preparing a modern textbook of psychiatry is the problem of correlating and integrating psychodynamic considerations with the descriptive delineation of reaction types. For many students this difficulty is somewhat increased in this textbook by the plan of organization in which the psychodynamics are somewhat segregated in the first part, dealing with normal behavior, and the second part is left rather starkly descriptive. For the more advanced student, prepared to utilize "genetic-dynamic" concepts in an integrated mastery of the materials presented, Dr. Muncie's book offers a treasury of case material, with many wise and penetrating comments, and a generous selection of pertinent references to American and European authors.

J. C. W.

Unipolar Lead Electrocardiography. By EMANUEL GOLDBERGER. Illus. 182 pp. $4.00. *Lea & Febiger, Philadelphia, Pennsylvania*, 1947.

Unipolar lead electrocardiography offers certain advantages over conventional methods. The conventional electrocardiographic leads are bipolar, recording the difference in potential between the two electrodes. Wilson in 1934 devised a unipolar lead so that the indifferent electrode has a theoretical potential of zero. Such a lead, therefore, represents primarily the potential at the exploring electrode, enabling one to record the potential at any one point rather than between two points on the body.

An increasing number of original articles attest to the growing knowledge in this comparatively new field. Goldberger's is the first text-book to be published on this subject. Much of this book is devoted to a detailed description of the electrocardiographic illustrations. Flagrant errors are noted when one attempts to check the descriptions with the actual electrocardiograms. Normal patterns are referred to as abnormal. Abnormal records are described as normal. The text description contradicts the labels under the illustrations. P and R waves described as of high voltage are actually within the normal limits quoted by the author. The author's chief difficulty seems to lie with the T waves, and he frequently describes as "downward" T waves which are actually upright, isoelectric or diphasic.

There are many vague, loose and incorrect statements. On page 61 the statement appears, "When the heart is vertical, $P_{L.arm}$ is frequently downward, but exceptions to this statement are common." However, in illustrations of 10 normal vertical hearts, $P_{L.arm}$ is downward in only 2, upright in 4, isoelectric in 4. In discussing left ventricular strain (page 94) one finds, "in this case, therefore, the presence of ventricular hypertrophy was indirectly shown by the electrocardiographic signs of auricular hypertrophy," (large P waves). Again, on page 100, "This tracing, therefore, only indirectly shows the presence of right ventricular hypertrophy due to the presence of the electrocardiographic patterns of auricular hypertrophy." The author twice states that when a myocardial infarct heals, the RS-T segment and T waves become normal. Not all cardiographers will accept this or many other statements in this text.

The book is poorly organized. The only illustration and description of a unipolar electrocardiogram in the section on congenital cardiovascular disease is that of dextrocardia. Insufficient evidence is offered for many of the statements in this book.

This volume fails to cover the subject thoroughly or accurately. It is, therefore, not recommended. There is still a need for a good text-book on unipolar electrocardiography.

S. S.